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Anti-diabetic and anti-adipogenic effects of a novel selective 11β-hydroxysteroid dehydrogenase type 1 inhibitor, 2-(3-benzoyl)-4-hydroxy-1,1-dioxo-2H-1, 2-benzothiazine-2-yl-1-phenylethanone (KR-66344)

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ABSTRACT

The selective inhibitors of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) have considerable potential for treating type 2 diabetes mellitus and metabolic syndrome. In the present study, we investigated the anti-diabetic and anti-adipogenic effects of 2-(3-benzoyl)-4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-2-yl-1-phenylethanone (KR-66344), as a 11β-HSD1 inhibitor; we also investigated the underlying molecular mechanisms in the cortisone-induced 3T3-L1 adipogenesis model system and C57BL/6-Lep$^{ob/ob}$ mice. KR-66344 concentration-dependently inhibited 11β-HSD1 activity in human liver microsome, mouse C2C12 myotube and human SW982 cells. In the C57BL/6-Lep$^{ob/ob}$ mice study, the administration of KR-66344 (200 mg/kg/d, orally for 5 days) improved the glucose intolerance as determined by the oral glucose tolerance test, in which the area under the curve (AUC) of the plasma glucose concentration was significantly reduced by 27% compared with the vehicle treated group. Further, KR-66344 suppressed adipocyte differentiation on cortisone-induced adipogenesis in 3T3-L1 cells is associated with the suppression of the cortisone-induced mRNA levels of FABP4, G3PD, PPARγ2 and Glut4, and 11β-HSD1 expression and activity. Our results additionally demonstrate evidence showing that KR-66344 improved glycemic control and inhibited adipogenesis via 11β-HSD1 enzyme activity. Taken together, these results may provide evidence of the therapeutic potential of KR-66344, as a 11β-HSD1 inhibitor, in obesity and type 2 diabetes patients with metabolic syndrome.

1. Introduction

It has been reported that glucocorticoid excess (Cushing's syndrome) causes metabolic syndrome such as visceral obesity, insulin resistance, diabetes mellitus, dyslipidaemia and hypertension. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) as an endoplasmic reticulum-associated enzyme of a NADPH dependent reductase converts inactive cortisone to the active glucocorticoid cortisol, and is highly expressed in liver and adipose tissues.

The relationship between 11β-HSD1 and type 2 diabetes mellitus (T2DM) has been demonstrated in mouse genetic models. The 11β-HSD1-transgenic mice in adipose tissue showed metabolic syndrome-like phenotypes such as central obesity, glucose intolerance, and insulin resistance [1]. In contrast, 11β-HSD1 knock mice showed a reduction in high-fat diet-induced obesity with improved insulin sensitivity and lipid profiles [2]. Based on these facts, 11β-HSD1 was considered as a drug target for the treatment of metabolic syndrome as well as T2DM.

Carbenoxolone, a non-selective 11β-HSD1 inhibitor, decreases glycogenolysis in diabetic patients, likely due to the inhibition of 11β-HSD2 [3]. BVT.2733, the 11β-HSD inhibitor of Biovitrum, lowered glucose and insulin levels and expression levels of mRNA encoding for PEPCK and G6Pase in liver tissue [4] and another inhibitor of Merck was reported to improve insulin sensitivity and to decrease the food intake and body weight gain in streptozotocin treated DIO mice [5]. Recently, Pfizer's aminopyridine analogue PF-915275 significantly reduced plasma insulin levels and showed inhibition of 11β-HSD1-mediated biomarker conversion using exogenous prednisone in cynomolgus monkeys [6]. Moreover, Incyte's INCB-13739 and Amgen's AMG-221 is currently in phase II or phase I clinical trials.

Recent papers showed that 11β-HSD1 inhibitors prevent adipogenesis and may be beneficial for the treatment of obesity in diabetic patients [7]. Glucocorticoids are essential for terminal adipogenesis of preadipocytes [8]. Abundant evidence accumu-

Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; G3PD, glycerol-3-phosphate dehydrogenase; PPARγ, peroxisome proliferator-activated receptor γ; FABP4, fatty acid-binding protein 4; GLUT4, glucose transporter 4.

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lates that mature adipocytes express late differentiation genes involved in lipid metabolism and lipid transport; glycerol-3-phosphate dehydrogenase (G3PD; [9,10]), peroxisome proliferator-activated receptor γ (PPARγ; [9]), fatty acid-binding protein 4 (FABP4; [11]) and glucose transporter 4 (GLUT4; [12]). Many of these genes have been reportedly regulated by glucocorticoids [13,14].

We previously reported that KR-66344, 2-(3-benzoyl)-4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-2-yl-1-phenylethanone, acts as a novel 11β-HSD1 inhibitor. This compound showed a potent *in vitro* activity against human 11β-HSD1 with IC_{50} values of 31 nM, and exhibited no binding with hERG. Additionally, the rat PK profiles of KR-66344 showed good oral bioavailability [15].

In the present study, we have explored whether KR-66344 can improve glucose tolerance in C57BL/6-Lep$^{ob/ob}$ mice and additionally prevent the cortisone-induced adipogenesis via the inhibition of 11β-HSD1 enzyme activity. In addition, KR-66344 has provided an anti-adipogenesis mechanism against cortisone-induced adipogenesis by suppression of adipogenic marker gene expressions such as FABP4, G3PD, Glut4 and PPARγ.

2. Materials and methods

2.1. Animals and drug administration

All of the animals were maintained in a room illuminated daily from 07:00 to 19:00 (12:12 h light/dark cycle), with controlled temperature (23 ± 1 °C) and ventilation (10–12 times per hour), and humidity was maintained at 55 ± 5%. Mice were caged individually and allowed free access to food and tap water in accordance with the *Guide for the Care and Use of Laboratory Animal* (NIH). All animal experiments were carried out with 7–9-week-old male C57BL/6J Lep$^{ob/ob}$ (*ob/ob*) mice and lean mice littermates according to the Guidelines for Animal Experimentation under admission of *the Institutional Animal Care and Use committee* (IACUC of KRICT).

Male *ob/ob* mice were orally gavaged with vehicle (0.5% CMC in H_2O). Reference compounds, BVT^{2733} and BVT^{14225}, and KR-66344 (200 mg/kg body weight) were administrated by oral gavage daily at 17:00 to 18:00 for 5 days. Animals were weighed regularly to allow accurate dosing with drugs. Plasma concentrations of glucose, triglyceride (TG), free fatty acid (FFA), HLD-cholesterol, and LDL-cholesterol were measured by a colorimetric assay using an automatic biochemical analyzer, the Selectra 2 (Vital Scientific N.V., The Netherlands).

2.2. The oral glucose tolerance test (OGTT) assay

Animals were weighed regularly to allow accurate dosing with drugs. All mice were fasted for 16 h and basal blood samples were collected from the ophthalmic venous plexus using heparin-coated capillary tubes. The mice were orally administered with glucose (2 g/kg) and additional blood samples were obtained at 15, 30, 60 and 120 min after glucose treatment. The blood samples were immediately centrifuged for 10 min at 1000 × *g* and the resulting plasma samples were stored at −20 °C until assayed. The plasma concentration of glucose was measured by colorimetric assay using an automatic biochemical analyzer, the Selectra 2 (Vital Scientific N. V., The Netherlands).

2.3. Cell culture and differentiation

All cell lines used in the experiment were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). C2Cl2 cells (ATCC CRL-1772, mouse skeletal muscle cell line) and 3T3-L1 cells (ATCC CL-173, mouse adipocyte) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin. SW982 cells (ATCC HTB-93, human synovial fibroblast cell line) were maintained with L-15 medium supplemented with 10% heat inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

C2C12 cells were induced to differentiate into myotubes by changing complete medium into a differentiation medium containing 2% fetal bovine serum and were allowed to grow further for at least 4 days.

The 3T3-L1 cells differentiated to mature adipocytes by the same medium containing 0.5 μM cortisone, 20 μg/ml insulin, 0.5 mM isobutylmethylxanthine (IBMX) and 1 μM dexamethasone for 3 days, which was then replaced with medium containing 0.5 μM cortisone and 20 μg/ml insulin for 2 days, and the cells were then cultured for 1 day in culture medium. To define the effect of the KR-66344 inhibitor upon the differentiation of preadipocytes, cultures were supplemented with 0.1–20 μM KR-66344. Media were replenished and KR-66344 or vehicle treatment repeated every 2–3 days.

2.4. In vitro assay for 11β-HSD1 activity

To assay microsomal 11β-HSD1 activity, 10 μg human microsome was added in an assay buffer (100 μl) containing 250 μM NADPH, 160 nM cortisone, 20 mM Tris–HCl, and 5 mM ethylenediaminetetraacetic acid (EDTA, pH 6.0) with or without compounds (in dimethyl sulfoxide [DMSO], final 1%) and allowed to incubate for 3 h at 37 °C. Small aliquots (2 μl) of the reaction mixtures were removed and subjected to HTRF cortisol assay according to the manufacturer's instructions (Nihon Schering, Tokyo, Japan). The HTRF assay is based on a competition between free cortisol and XL665-conjugated cortisol for the binding to an anticortisol antibody labeled with europium (Eu^{3+}) cryptate. Eu^{3+} cryptate and XL665 act as donor and acceptor, respectively. If the two fluorophores will be in close proximity, fluorescence resonance energy transfer (FRET) occurs on excitation. A specific signal is expressed as percentage of Delta *F*, which is a value calculated from the ratio of 665 nm/615 nm [$(R_{sample} - R_{negative})/R_{negative} \times 100$], and is inversely proportional to the concentration of cortisol in the sample or the calibrator. The cortisol concentration was calculated from the calibration curve obtained from Delta *F* versus standard solution. IC_{50} values of compounds were determined from concentration-dependent inhibition curves by GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Cellular 11β-HSD1 enzyme activity was measured by the HTRF cortisol assay kit. Myotubes, SW982 cells or adipocytes were seeded at 1×10^5 cells/well, 5×10^5 cells/well or 5×10^4 cells/well onto 24-well plates and were incubated in a medium containing 160 nM cortisone (total 1 ml) in the presence or absence of compounds. Samples of the medium (2 μl) were taken at 24 h, and the enzyme activity was assessed by the HTRF method.

Carbenoxolone, BVT 14225 and BVT 2733 were used the reference compounds.

2.5. Oil red O staining and quantification of lipid content

The cellular lipid content was assessed by Oil Red O staining (sigma, MO, USA). At 6 days, cells were washed and fixed in 4% formalin for 1 h, stained with Oil Red O working solution and incubated for another 1 h at room temperature. After being washed thrice with PBS, the cells were photographed by a light microscope (Olympus, Japan). To assay for the intracellular lipid content, each well was added with 300 μl isopropanol. After incubation at room temperature for 20 min, the optical density was measured at

Table 1
Primer sequences for various mouse genes for real-time PCR.

Gene	Forward	Reverse
11β-HSD1	gggataattgacgccctagc	tgaggcaggactgttctaag
FABP4	catcagcgtaaatggggatt	tcgactttccatcccacttc
G3PD	agagatgctcgccacagaat	aaagggtctctggggtctgt
PPARγ2	ccctggcaaagcatttgtat	gaaactggcacccttgaaaa
Glut4	ctccttctatttgccgtcctc	ctgttttgcccctcagtcatt
GAPDH	aactttggcattgtggaagg	cacattgggggtaggaacac

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3).

450 nm using the microplate reader (Bio-Rad Laboratories, Hercules, CA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR analysis

Total RNA was isolated from compounds-treated cells and tissues using the easy-BLUE Total RNA extraction Kit (iNtRON Inc., Korea). Reverse transcription of total RNA (1 μg) was performed using AccuPower RT PreMix (Bioneer Inc., Korea).

Expression studies were carried out using gene specific primers for human 11β-HSD1, FABP4, G3PD, glucose transporter 4 (Glut4) and PPARγ. All primers were designed using primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) and their sequences from 5′ to 3′ are shown in Table 1.

Quantitative mRNA expression levels of 11β-HSD1, FABP4, G3PD, Glut4 and PPARγ2 were measured by real-time PCR using the Roter-Gene 3000 system (Corbett research, Australia). PCR was performed in 20 μl reactions. Reactions contained QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA), 2 μl of 10 pmol primers, 10 μl SYBR Green PCR probe and 1 μl cDNA. Reactions were as follows: 95 °C for 15 min, and then 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. The relative abundance of mRNA was calculated after normalization to GAPDH.

2.7. Drugs

KR-66344 and reference compounds were synthesized in the Korea Research Institute of Chemical Technology (Daejon, Korea). Carbenoxolone was purchased from Amifinecom Inc. (IL). Oil red O, NADPH and cortisone were from Sigma. Compounds were dissolved in dimethyl sulfoxide as a 20 mM stock solution and then diluted with phosphate buffered saline.

2.8. Statistics

The results are expressed as means ± S.E.M. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparison test. $P < 0.05$ was considered to be statistically significant for all experiments.

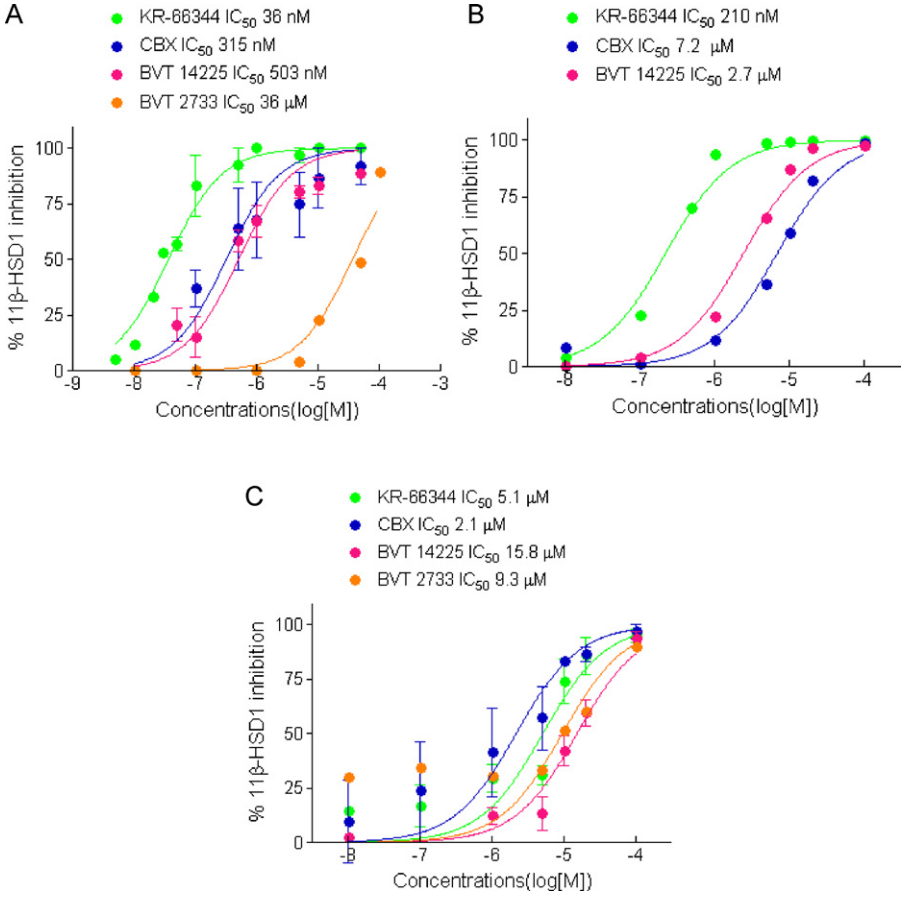

Fig. 1. *In vitro* 11βHSD1 enzyme activity in human liver microsome (A), mouse myotube cells (B) and human SW982 cells (C). (A) 10 μg human microsome was added in an assay buffer (100 μl) containing 250 μM NADPH, 160 nM cortisone, 20 mM Tris–HCl, and 5 mM ethylenediaminetetraacetic acid (EDTA, pH 6.0) with or without compounds (in dimethyl sulfoxide [DMSO], final 1%) and allowed to incubate for 3 h at 37 °C. (B and C) differentiated C2C12 and SW982 cells seeded at 1×10^5 cells/well and 5×10^5 cells/well onto 24-well plates and were incubated in a medium containing 160 nM cortisone (total 1 ml) in the presence or absence of compounds for 24 h at 37 °C. Results are expressed as means ± S.E.M. of triplicate experiments.

3. Results

3.1. Inhibitory effect of KR-66344 on human and mouse 11β-HSD1 enzyme activity

We assessed the *in vitro* 11β-HSD1enzyme activity by the HTRF method. Carbenoxolone, BVT 14225 and BVT 2733 were used as reference compounds. BVT 14225 and BVT 2733 are isoform inhibitors of human and mouse 11β-HSD1, respectively [16,17]. When the 11β-HSD1 enzyme activity was assayed in the human liver microsome incubation with KR-66344 for 3 h, it significantly inhibited the oxo-reductase activity of 11β-HSD1 (from cortisone to cortisol conversion) in a concentration-dependent manner with IC_{50} of 36 nM (Fig. 1A). The IC_{50} value of carbenoxolone, BVT 14225 and BVT 2733 was 315 nM, 503 nM and 36 μM.

Furthermore, KR-66344 inhibited the 11β-HSD1 activity in mouse C2C12 cells and human SW982 cells in a concentration dependent manner and the mouse and human IC_{50} values of KR-66344 were 210 nM and 5.1 μM, respectively (Fig. 1B and C). However, KR-66344 had no effect on 11β-HSD2 activity (6% inhibition in 20 μM, cortisol to cortisone conversion) in the human 11β-HSD2-overexpressed HEK293 cells (data not shown). No toxic effects of KR-66344 were observed up to 20 μM concentrations (data not shown). These results suggest that KR-66344 concentration-dependently inhibits 11β-HSD1 enzyme activity in human and mouse cell lines.

3.2. Effect of KR-66344 on glucose tolerance in ob/ob mice

The *ob/ob* mice received KR-66344 (200 mg/kg/d, orally for 5 days) to assess whether the inhibitory activity of KR-66344 on the 11β-HSD1 could ameliorate glucose intolerance. Oral treatment with KR-66344 showed increased glucose clearance. Plasma glucose levels, as determined by the AUC of the glucose concentration curve, were reduced by 27.02% compared to vehicle by administration of KR-66344 200 mg/kg (Fig. 2). The reference compound, BVT 2733, an isoform-selective inhibitor of mouse 11β-HSD1, was reduced by 22.26% compared to controls.

Moreover, treatment of KR-66344 improved plasma lipid profiles such as cholesterol, TG, HDL-cholesterol, glucose, FFA and LDL-cholesterol in *ob/ob* mice after 5 days (Table 2). The results suggested that KR-66344 could improve glucose tolerance and lipid profiles via inhibition of 11β-HSD1 enzyme activity.

3.3. Effect of KR-66344 on lipid accumulation and expression of adipogenic markers in adipocytes

The effect of KR-66344 on lipid accumulation was determined by Oil Red O staining (Fig. 3A). Preadipocytes with cortisone at various concentrations (0.01, 0.1, 0.5 and 1 μM) showed increased lipid accumulation when assessed after 6 days. These effects were abolished by co-incubation with KR-66344 in a concentration-dependent manner with 10 μM of KR-66344 being most effective ($P < 0.01$) (Fig. 3B).

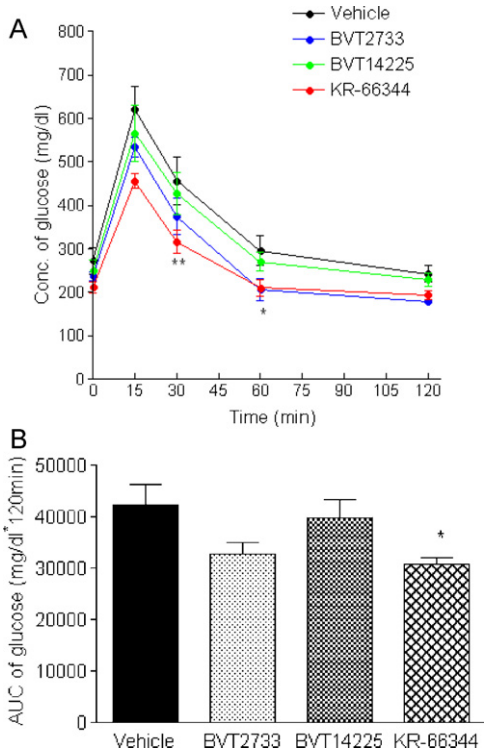

Fig. 2. Anti-diabetic efficacy study of KR-66344 in *ob/ob* mice. *ob/ob* mice were administered with BVT 2733, BVT 14225 and KR-66344 (200 mg/kg body weight) daily by oral gavage for 5 days. Compounds were administered orally to *ob/ob* mice, and glucose (2 g/kg, p.o.) was given 30 min later (0 min). Plasma glucose concentration was then measured at the times indicated. (A) (●) vehicle, (▲) BVT 2733, (■) BVT 14225, (◆) KR-66344. Results are expressed as means ± S.E.M. for $n = 7$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group.

Next, we examined whether the mRNA expression of adipogenesis-specific genes is inhibited by KR-66344. 3T3-L1 preadipocytes were treated with KR-66344 from day 0 to day 6. On day 6, real-time PCR was performed to analyze the mRNA levels of FABP4, G3PD, PPARγ2 and Glut4. As shown in Fig. 4, KR-66344 inhibited the expression of all these genes and achieved the maximal effect at the concentration of 10 μM.

Moreover, KR-66344 inhibited the 11β-HSD1 mRNA expression and enzyme activity in differentiated 3T3-L1 cells in a concentration-dependent manner (Fig. 5). The results suggested that KR-66344 could inhibit adipogenesis in 3T3-L1 adipocytes via the inhibition of 11β-HSD1 enzyme activity.

3.4. The binding mode for KR-66344 in human and mouse 11β-HSD1

The binding mode for KR-66344 was shown for human and mouse 11β-HSD1 in Fig. 6. The used reference structure for 11β-HSD1 complex was obtained from the protein data bank (pdb entry; 3FRJ [18] for human, 1Y5 M [19] for mouse). The calculation

Table 2
Metabolic parameters of mice with BVT2733, BVT14225 and KR-66344.

	Vehicle	BVT2733 (200 mg/kg)	BVT14225 (200 mg/kg)	KR-66344 (200 mg/kg)
Cholesterol (mg/dl)	184.9 ± 28.9	183.2 ± 13.1	188.7 ± 30.3	169.7 ± 8.8
TG (mg/dl)	109.7 ± 33.4	112.2 ± 12.7	108.2 ± 30.7	81.6 ± 10.5
HDL (mg/dl)	143.7 ± 27.2	140.0 ± 15.6	142.0 ± 30.6	126.2 ± 8.1
Glucose (mg/dl)	291.5 ± 68.5	236.5 ± 24.4	249.3 ± 61.7	195.9 ± 26.6*
FFA (μEq/L)	1428.5 ± 162.8	1580.3 ± 336.2	1319.6 ± 158.1	1126.1 ± 160.0*
LDL (mg/dl)	44.1 ± 3.9	41.5 ± 2.7	42.3 ± 5.2	45.8 ± 3.7

TG; triglyceride, HDL; high-density lipoprotein, FFA; free fatty acid, LDL; low-density lipoprotein. Values are mean ± SEM; $n = 5$–9/group.
* $P < 0.05$ vs vehicle group.

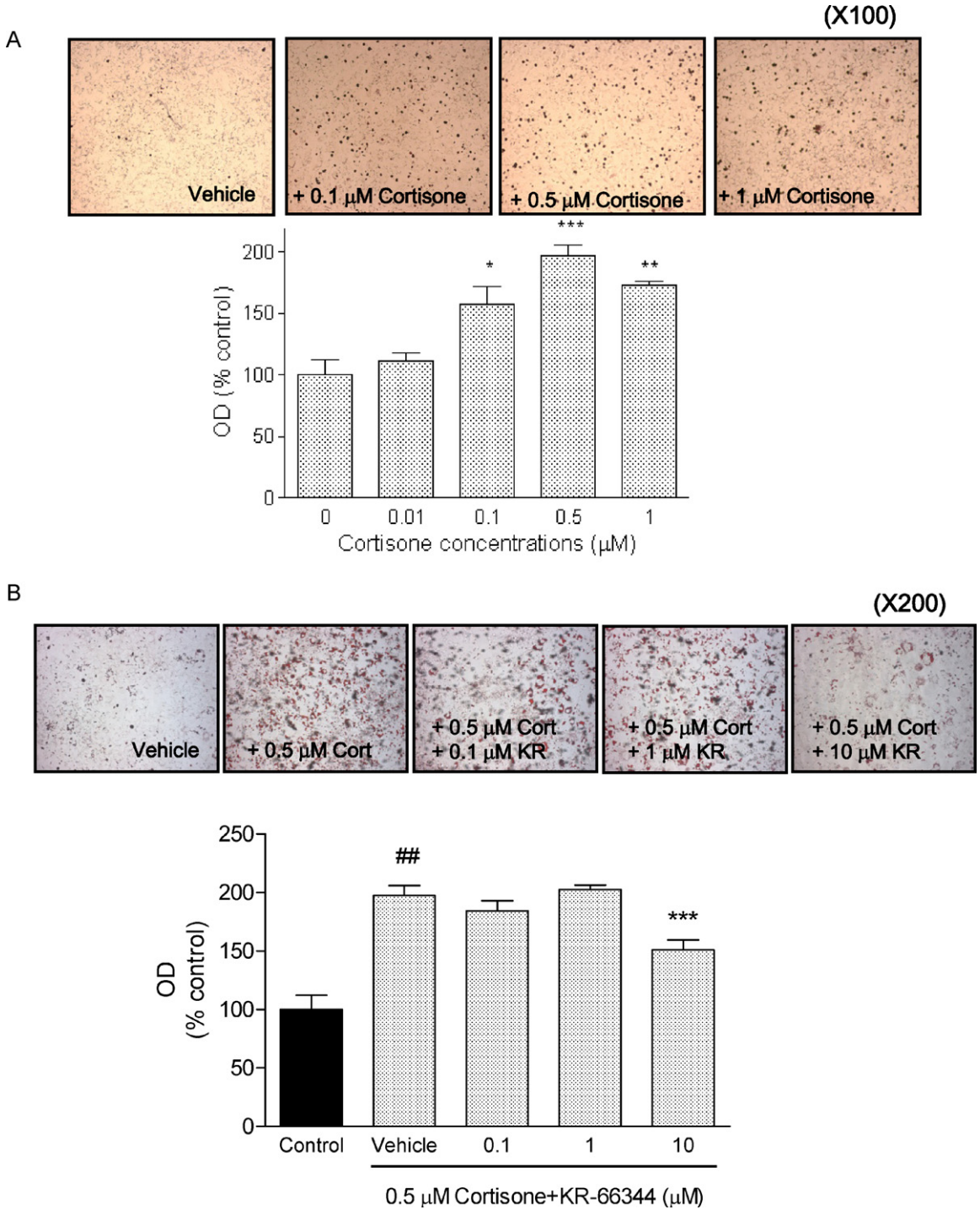

Fig. 3. Anti-adipogenic effect of KR-66344 on lipid accumulation in cortisone-treated 3T3-L1 cells. (A) Effect of cortisone on lipid accumulation and adipogenensis in 3T3-L1 cells. Preadipocytes were induced to differentiate with contisone in increasing concentrations for 6 days. The cellular lipid content was assessed by Oil Red O staining, and quantified by isopropanol. (B) Anti-adipogenic effect of KR-66344 in cortisone-treated 3T3-L1 cells. Preadipocytes were induced to differentiate with cortisone (0.5 μM) and KR-66344 in increasing concentrations for 6 days. Values are means ± S.E.M. of data from two separate experiments with three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle group.

for docking was carried out using LigandFit [20] interfaced with Accelrys DiscoveryStudio2.5. KR-66344 interacts through H-bonding with S170 and Y183 residues in the active sites in 11β-HSD1, and also shows the hydrophobic interaction with Y177 in human form. In mouse 11β-HSD1, Y284 residues in β-chain formed the sterically unfavorable interaction, and thus was considered to show low activity.

4. Discussion

11β-HSD1 is an endoplasmic reticulum-associated enzyme that acts as an NADPH dependent reductase and converts inactive cortisone to the active glucocorticoid cortisol. 11β-HSD1 is highly expressed in liver and adipose tissues. Recently, 11β-HSD1 has been recognized as a potential therapeutic target for the treatment

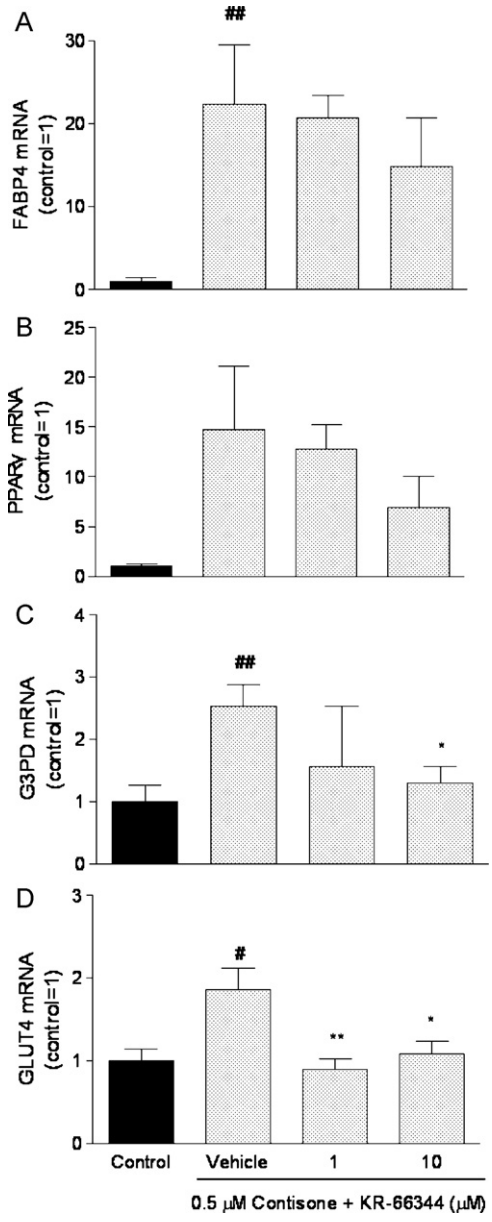

Fig. 4. Effect of KR-66344 on adipogenesis related gene expression in cortisone-treated 3T3-L1 cells. Preadipocytes were induced to differentiate with cortisone (0.5 μM) and KR-66344 (1 and 10 μM) for 6 days, adipogenesis marker expressions were determined by real-time PCR. Values are means ± S.E.M. of data from two separate experiments with six replicates. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. preadipocytes; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle group.

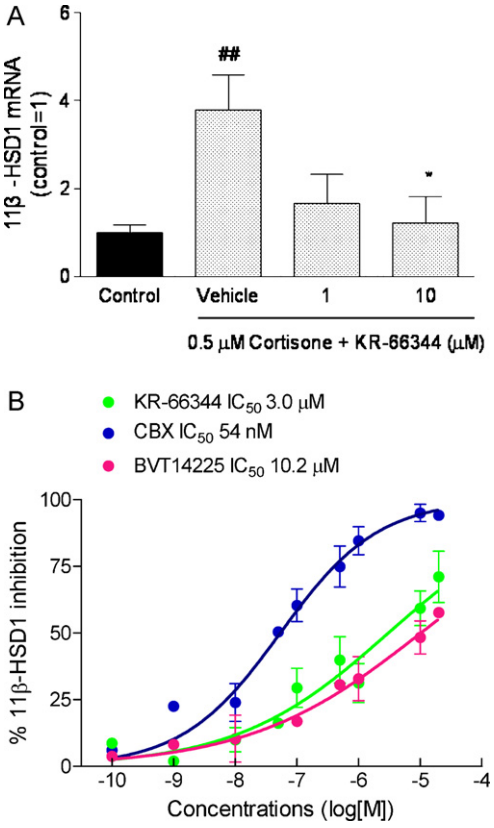

Fig. 5. *In vitro* 11β-HSD1 mRNA expression (A) and enzyme activity (B) in mouse 3T3-L1 adipocytes. (A) Preadipocytes were induced to differentiate with cortisone (0.5 μM) and KR-66344 (1 and 10 μM) for 6 days, 11β-HSD1 mRNA expression was determined by real-time PCR. Values are means ± S.E.M. of data from two separate experiments with six replicates. $^{\#\#}P < 0.01$ vs. preadipocytes; $^{*}P < 0.05$ vs. vehicle group. (B) Adipocytes seeded at 5×10^4 cells/well onto 24-well plates and were incubated in a medium containing 160 nM cortisone (total 1 ml) in the presence or absence of compounds. Results are expressed as means ± S.E.M. of triplicate experiments.

of type 2 diabetes with metabolic syndrome since various 11β-HSD1 inhibitors improved the glucose tolerance in the diabetic mice model [1,4] and inhibited adipogenesis and atherosclerosis. In the present study, we performed an *in vitro* and *in vivo* study to assess the anti-diabetic and anti-adipogenic effects of an 11β-HSD1 inhibitor, KR-66344 on the *ob/ob* diabetic mice model and cortisone-induced adipogenesis model.

The relationships between 11β-HSD1 and type 2 diabetes have been demonstrated in mouse genetic models The adipose tissues from 11β-HSD1-overexpressing transgenic mice showed metabolic syndrome-like phenotypes such as central obesity, glucose intolerance, and insulin resistance [1], but those tissues from 11β-HSD1 knock mice showed a reduction in high-fat diet-induced obesity with improved insulin sensitivity and lipid profiles [2]. Moreover, a number of small molecule 11β-HSD1 inhibitors showed efficacy in rodent models related to diabetes. In the previous study, we reported that KR-66344, 2-(3-benzoyl)-4-hydroxy-1,1-dioxo-2H-1,2-benzothiazine-2-yl-1-phenylethanone, acted as a novel 11β-HSD1 inhibitor and KR-66344 revealed a very low IC_{50} value (31 nM) against human 11β-HSD1 [15]. In this study, KR-66344 inhibited the 11β-HSD1 activity in mouse C2C12 cells and human SW982 cells in a concentration-dependent manner, but KR-66344 was without effect on 11β-HSD2 activity in human 11β-HSD2-overexpressing HEK293 cells (data not shown). These results indicate that KR-66344 concentration-dependently inhibits 11β-HSD1 enzyme activity in human and mouse cell lines.

To examine whether the inhibitory activity of KR-66344 on 11β-HSD1 could ameliorate glucose intolerance, the effect of KR-66344 on glucose tolerance was tested using *ob/ob* mice via an OGTT. Oral treatment with KR-66344 (200 mg/kg/d for 5 days) exhibited significantly increased glucose clearance associated with reduced plasma glucose levels, as contrasted to the reference compounds, BVT 2733. Furthermore, treatment of KR-66344 associated with improved plasma lipid profiles such as cholesterol, TG, HDL-cholesterol, glucose, FFA and LDL-cholesterol in *ob/ob* mice after 5 days. By comparison, the effects of BVT 2733 were examined, and its effects were weaker than that of KR-66344. Considering these previous results, it is suggested that improvement of glucose tolerance and plasma lipid profiles by KR-66344 in *ob/ob* diabetic mice is ascribed to the inhibition of 11β-HSD1 enzyme activity.

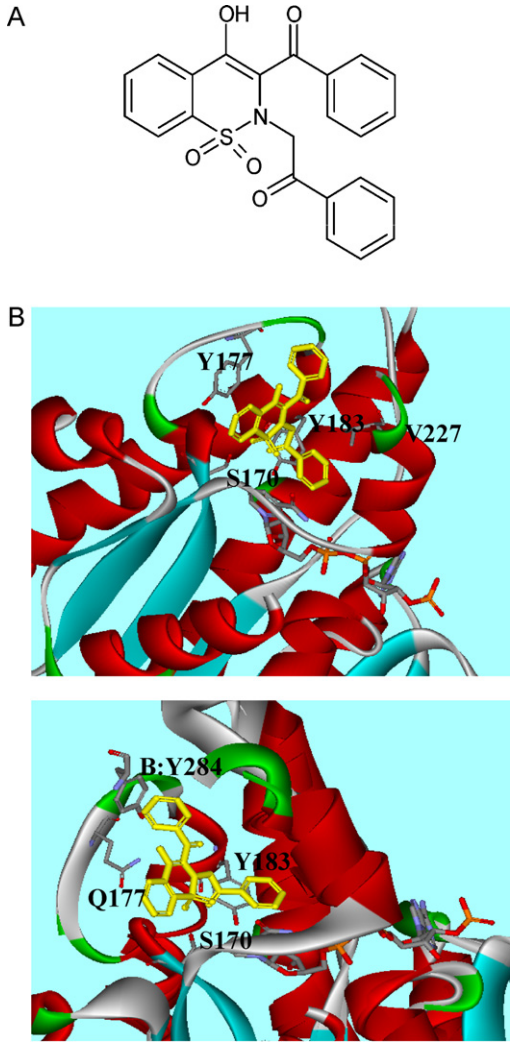

Fig. 6. Structure (A) and the binding mode (B) of KR-66344. (B) The binding mode of KR-66344 compound was shown for human (upper) and mouse (lower) 11β-HSD1. The important residues for the interaction are represented by one-letter codes. KR-66344 is shown in yellow and cofactor NADP in elemental colors by stick. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

Recent investigations showed that 11β-HSD1 inhibitors prevented adipogenesis with beneficial application to the treatment of obesity in diabetic patients [7]. Mature adipocytes express late differentiation genes involved in lipid metabolism and lipid transport, including G3PD and FABP4 [11], and many of these genes are regulated by glucocorticoids [13,14]. PPARγ was shown to play an important role in adipocyte differentiation, glucose homeostasis and insulin signaling [21]. Moreover, Glut4 is selectively expressed in insulin-sensitive tissues such as muscle and adipose cells [22] and impairment of glucose uptake by adipose and muscle tissues in obesity is associated with the reduction of cellular Glut4 content [23], while a loss of body weight from diet therapy greatly improves abnormal glucose homeostasis [24]. In our results, the effect of KR-66344 on lipid accumulation was measured by Oil Red O staining. Preadipocytes with cortisone at various concentrations (0.01, 0.1, 0.5 and 1 μM) showed increased lipid accumulation for 6 days. These effects were completely abolished by co-incubation with KR-66344 in a dose-dependent manner, and the most effective dosage for inhibition was 10 μM of KR-66344. In line with these, the mRNA expression of adipogenesis-specific genes was inhibited by KR-66344; the mRNA levels of FABP4, G3PD, PPARγ2, and Glut4 was increased by 0.5 μM cortisone; these inductions were inhibited by 10 μM KR-66344. Moreover, KR-66344 inhibited the 11β-HSD1 mRNA expression and enzyme activity in differentiated 3T3-L1 cells. These results provide evidence to support our hypothesis that KR-66344 can inhibit cortisone-induced adipogenesis in 3T3-L1 adipocytes via inhibition of 11β-HSD1 expression and enzyme activity.

We also identified the binding mode for KR-66344 for human and mouse 11β-HSD1. KR-66344 interacts through H-bonding with S170 and Y183 residues in the active sites in 11β-HSD1, and also shows the hydrophobic interaction with Y177 in human form. In mice, Y284 residues in the β-chain formed the sterically unfavorable interaction, thus resulting in lowering activity.

In these studies, carbenoxolone was more potent than KR-66344 on the 11β-HSD1 enzyme activity in differentiated 3T3-L1 cells and C2C12 cells. However, carbenoxolone is a non-selective 11β-HSD1 inhibitor and tends to decrease glycogenolysis in diabetic patients, likely due to the inhibition of 11β-HSD2 [3]. Furthermore, KR-66344 has more 11β-HSD1 inhibition potency than carbenoxolone in human microsome and human SW982 cells and the effects of selective inhibitors, BVT 14225 and BVT 2733, were weaker than that of KR-66334 on 11β-HSD1 enzyme activity in human and mouse cell lines and OGTT assay in *ob/ob* mice. Moreover, our previous study showed that KR-66344 exhibited little binding with hERG and the rat PK profiles of KR-66344 showed good oral bioavailability [15]. Taken all together, it is concluded that a selective 11β-HSD1 inhibitor, KR-66344, may provide a new strategy in the prevention and treatment of type 2 diabetes patients with obesity without toxicity.

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References

[1] Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, et al. A transgenic model of visceral obesity and the metabolic syndrome. Science 2001;294:2166–70.
[2] Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, et al. Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice. Diabetes 2004;53:931–8.
[3] Andrews RC, Rooyackers O, Walker BR. Effects of the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes. J Clin Endocrinol Metab 2003;88:285–91.
[4] Alberts P, Engblom L, Edling N, Forsgren M, Klingström G, Larsson C, et al. Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. Diabetologia 2002;45:1528–32.
[5] Hermanowski-Vosatka A, Balkovec JM, Cheng K, Chen HY, Hernandez M, Koo GC, et al. 11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. J Exp Med 2005;202:517–27.
[6] Bhat BG, Hosea N, Fanjul A, Herrera J, Chapman J, Thalacker F, et al. Demonstration of proof of mechanism and pharmacokinetics and pharmacodynamic relationship with 4′-cyano-biphenyl-4-sulfonic acid (6-amino-pyridin-2-yl)-amide (PF-915275), an inhibitor of 11 -hydroxysteroid dehydrogenase type 1, in cynomolgus monkeys. J Pharmacol Exp Ther 2008;324:299–305.
[7] Bujalska IJ, Gathercole LL, Tomlinson JW, Darimont C, Ermolieff J, Fanjul AN, et al. A novel selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor prevents human adipogenesis. J Endocrinol 2008;197:297–307.
[8] Hauner H, Schmid P, Pfeiffer EF. Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. J Clin Endocrinol Metab 1987;64:832–5.
[9] Hirai S, Yamanaka M, Kawachi H, Matsui T, Yano H. Activin A inhibits differentiation of 3T3-L1 preadipocyte. Mol Cell Endocrinol 2005;232:21–6.
[10] Brandebourg TD, Hu CY. Isomer-specific regulation of differentiating pig preadipocytes by conjugated linoleic acids. J Anim Sci 2005;83:2096–105.
[11] Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science 1996;274:1377–9.
[12] Park SY, Choi GH, Choi HI, Ryu J, Jung CY, Lee W. Calorie restriction improves whole-body glucose disposal and insulin resistance in association with the

increased adipocyte-specific GLUT4 expression in Otsuka Long-Evans Tokushima fatty rats. Arch Biochem Biophys 2005;436:276–84.
[13] Wu Z, Bucher NL, Farmer SR. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. Mol Cell Biol 1996;16:4128–36.
[14] Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 2006;7:885–96.
[15] Kim SH, Ramu R, Kwon SW, Lee SH, Kim CH, Kang SK, et al. Discovery of cyclicsulfonamide derivatives as 11 beta-hydroxysteroid dehydrogenase 1 inhibitors. Bioorg Med Chem Lett 2010;20:1065–9.
[16] Vicker N, Su X, Ganeshapillai D, Smith A, Purohit A, Reed MJ, et al. Novel non-steroidal inhibitors of human 11beta-hydroxysteroid dehydrogenase type 1. J Steroid Biochem Mol Biol 2007;104:123–9.
[17] Alberts P, Nilsson C, Selen G, Engblom LO, Edling NH, Norling S, et al. Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. Endocrinology 2003;144:4755–62.
[18] Rew Y, McMinn DL, Wang Z, He X, Hungate RW, Jaen JC, et al. Discovery and optimization of piperidyl benzamide derivatives as a novel class of 11beta-HSD1 inhibitors. Bioorg Med Chem Lett 2009;19:1797–801.
[19] Zhang J, Osslund TD, Plant MH, Clogston CL, Nybo RE, Xiong F, et al. Crystal structure of murine 11 beta-hydroxysteroid dehydrogenase 1: an important therapeutic target for diabetes. Biochemistry 2005;44:6948–57.
[20] Venkatachalam CM, Jiang X, Oldfield T, Waldman M. LigandFit: a novel method for the shape-directed rapid docking of ligands to protein active sites. J Mol Graph Model 2003;21:289–307.
[21] Kersten JR, Toller WG, Gross ER, Pagel PS, Warltier DC. Diabetes abolishes ischemic preconditioning: role of glucose, insulin, and osmolality. Am J Physiol Heart Circ Physiol 2000;278:H1218–24.
[22] Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. Nat Rev Mol Cell Biol 2002;3:267–77.
[23] Sato T, Man ZW, Toide K, Asahi Y. Plasma membrane content of insulin-regulated glucose transporter in skeletal muscle of the male Otsuka Long-Evans Tokushima fatty rat, a model of non-insulin-dependent diabetes mellitus. FEBS Lett 1997;407:329–32.
[24] Wing RR, Blair EH, Bononi P, Marcus MD, Watanabe R, Bergman RN. Caloric restriction per se is a significant factor in improvements in glycemic control and insulin sensitivity during weight loss in obese NIDDM patients. Diab Care 1994;17:30–6.