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Adiponectin receptor 2 expression in liver and insulin resistance in *db*/*db* mice given a β_3-adrenoceptor agonist

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Abstract

Our aim was to determine the effect of a β_3-adrenoceptor agonist on plasma adiponectin levels and on the level of expression of mRNA for adiponectin, adiponectin receptor 1, and adiponectin receptor 2 in *db*/*db* mice. Two weeks' oral administration of CL-316,243 led to decreased plasma levels of hemoglobin A1c, glucose, insulin, triglyceride and free fatty acid, and to an increased plasma adiponectin levels. It also improved insulin resistance in the oral glucose tolerance test. Adiponectin mRNA expression was significantly higher in the CL-316,243-treatment group than in the control group in epididymal white adipose tissue but not in brown adipose tissue, soleus muscle or liver. Adiponectin receptor 2 mRNA expression was significantly lower only in the liver of the CL-316,243-treatment group (versus the control group).

These results suggest that the increased plasma adiponectin levels seen in *db*/*db* mice treated with this β_3-adrenoceptor agonist induce a down-regulation of adiponectin receptor 2 mRNA expression specifically in the liver.
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Keywords: Adiponectin; Adiponectin receptor 1; Adiponectin receptor 2; β_3-aderenoceptor agonist

1. Introduction

The β-adrenoceptors belong to the category of G protein-coupled receptors containing seven membrane-spanning regions and mediate the physiological actions of adrenaline and noradrenaline by activating adenylyl cyclase via stimulatory G proteins (Pierce et al., 2002). Stimulation of β_1-adrenoceptors provokes an increase in heart rate, while stimulation of β_2-adrenoceptors mainly induces relaxation of bronchial smooth muscle. Adrenoceptors are found in white adipose tissue, which stores excess energy as triglycerides, and in brown adipose tissue, which contains a wealth of mitochondria and looks brown due to the presence of cytochrome. The finding of both an impaired expression and function of β_3-adrenoceptors in genetic and dietary obesity in mice and an increase in total body fat in β_3-adrenoceptor-deficient mice suggests a correlation between β_3-adrenoceptors and obesity (Susulic et al., 1995; Collins et al., 1999).

Many β_3-adrenoceptor agonists have been developed. One of them (AJ-9677), when given to KK-A^y/Ta mice, induced significant decreases in adipose tissue weight and in plasma glucose, insulin, free fatty acid and triglyceride levels, and also upregulated the expression of mRNA for uncoupling protein-1 in brown adipose tissue and white adipose tissue (Kato et al., 2001). Another (CL-316,243), when administered to Zucker diabetic fatty rats, decreased the plasma glucose and insulin levels and improved type 2 diabetes (Liu et al., 1998). Such results indicate a close correlation between a decreased function of β_3-adrenoceptors and both obesity and diabetes. The β_3-adrenoceptor agonists have the ability to improve metabolic syndrome and might become an attractive drug for it.

Adipose tissue not only functions as an energy-storage organ but also as an endocrine organ, secreting a wealth of different protein factors into the circulation. Adiponectin is

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an insulin-sensitive hormone and plays a central role in both glucose and lipid metabolism (Berg et al., 2002; Ukkola and Santaniemi, 2002; Haval, 2004). Plasma levels of adiponectin are known to be reduced in obese and diabetic rhesus monkeys and also in human type 2 diabetics (Hotta et al., 2001, 2000). However, nothing is known about the effects of β_3-adrenoceptor agonists on the plasma adiponectin level or on adiponectin receptors in *db*/*db* mice, which are genetically obese and diabetic.

The aim of this study with *db*/*db* mice was to determine the effects of a β_3-adrenoceptor agonist (CL-316,243) on plasma adiponectin levels and on the level of expression of mRNA for adiponectin, adiponectin receptor 1, and adiponectin receptor 2 in various tissues.

2. Materials and methods

2.1. Drug

(*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethylamino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL-316,243) was synthesized in our laboratories (Kissei, Hotaka, Japan).

2.2. Animal studies

Male 5-week-old C57BL/KsJ-*db*/*db* mice were purchased from Clea Japan (Tokyo, Japan). They were allowed free access to laboratory chow (CE-2; Oriental Yeast, Tokyo, Japan) and water. The treatment group was given CL-316,243 (3 mg/kg) by gavage daily at 9:00 AM for 2 weeks, with the control group being given 0.5% methyl cellulose solution. Blood samples were collected from the retro-orbital sinus by using Heparinized Calibrated Pipets (Drummond Scientific Company, Broomall, PA, USA), and plasma was prepared by centrifugation. The plasma hemoglobin A1c level was measured using the glycohemoglobin analyzer HLC-723GHbV from Tosoh Coporation (Yamaguchi, Japan). The plasma insulin level was determined using a Morinaga insulin assay kit (Morinaga Bioscience Laboratory, Yokohama, Japan), the plasma triglyceride level using a Triglyceride E Test (Wako Pure Chemical Industries, Osaka, Japan), the plasma glucose level using a Glucose C-II Test (Wako Pure Chemical Industries), the plasma free fatty acids level using a non-esterified fatty acid (NEFA) C-Test (Wako Pure Chemical Industries), and the plasma adiponectin level using a Mouse/Rat adiponectin ELISA Kit (Otsuka Pharmaceutical Co.,Ltd, Tokyo, Japan). An oral glucose tolerance test was performed on day 14 after a fast of 16 h. In this test, each mouse received 20% glucose solution (2 g/kg) and blood samples were collected just before and at 0.5, 1 and 2 h after glucose loading. All procedures involving the use of laboratory animals in this study, including blood sampling from veins and death caused by diethyl ether overdosing, were conducted according to guidelines approved by the Laboratory Animal Committee of Kissei Pharmaceutical Co., Ltd. Moreover, the protocols complied with European Community guidelines for the use of experimental animals.

2.3. Tissue RNA isolation

To quantify the expression of adiponectin, adiponectin receptor 1, and adiponectin receptor 2, mice were killed after 2 weeks of the above treatment. Epididymal white adipose tissue, interscapular brown adipose tissue, soleus muscle and liver were excised and weighed; tissue was stored at −80 °C. Each tissue was then homogenized for 1 min in TRIZOL® Reagent (Invitrogen, California, USA), using an Ultra-turrax T8 homogenizer (IKA-Werke GmbH and CO.KG, Staufen, Germany). Subsequent procedures were carried out according to the manufacturer's instructions unless otherwise specified. Isolated total RNA samples were cleaned up using an RNeasy® Mini Kit (Qiagen, Valencia, USA).

2.4. Reverse transcription and real-time quantitative polymerase chain reaction

Reverse transcription of 1 μg total RNA to cDNA was performed using a Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA) according to the manufacturer's instructions. Forward primer, reverse primer and Taqman probe were designed using Primer Express software version 1.0 (Applied Biosystems, Foster City, USA). Real-time PCR-based 5′ nuclease assays (Taqman assays) were performed by

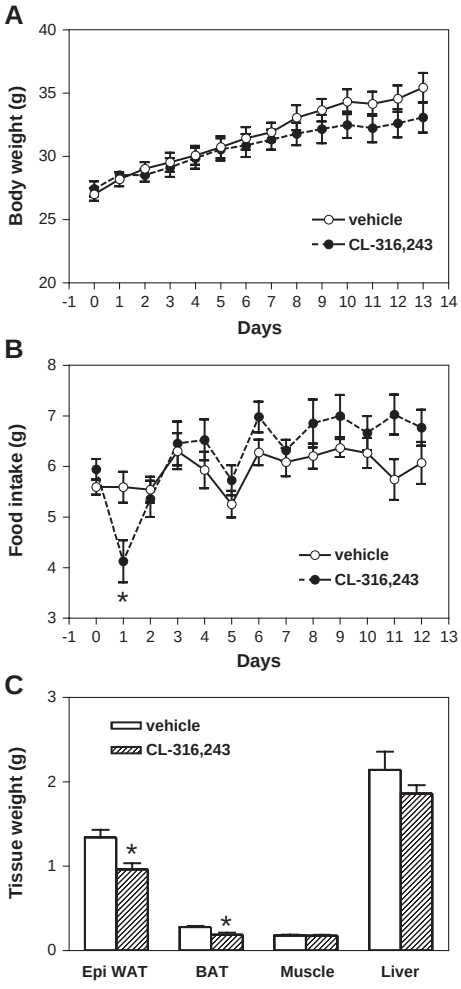

Fig. 1. Body weight, food intake and tissue weight in control and CL-316,243-treated *db*/*db* mice. A, body weight; B, food intake; C, tissue weight after 2 weeks' treatment. In C, Epi WAT is epididymal white adipose tissue, BAT is brown adipose tissue. Mean ± S.E.M.; $n = 5$ in each group. *$P < 0.05$ vs. control group.

using a Gene Amp® 5700 Sequence Detection System (Applied Biosystems). The following primers were used: adiponectin, forward 5′-TTTGGTCCCTCCACCCAAG-3′, reverse 5′-CCTTCTCTCCAGGAGTGCCAT-3′, fluorogenic probe FAM-5′-AGGTTGGATGGCAGGCATCCCA-3′-TAMRA; adiponectin receptor 1, forward 5′-ACGTTGGAGAGTCATCCCGTAT-3′, reverse 5′-TCTTGAAGCAAGCCCGAAAG-3′, fluorogenic probe FAM-5′-AAAGACAACGACTACCTGCTACATGGCCAC-3′-TAMRA; adiponectin receptor 2, forward 5′-AGCCTCTATATCACCGGAGCTG-3′, reverse 5′-GCTGATGAGAGTGAAACCAGATGT-3′, fluorogenic probe FAM-5′-CCTGAGCGCTTCTTTCCTGGCAAA-3′-TAMRA. Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Control Reagents (Applied Biosystems) were used as primers for mouse GAPDH. Numbers of cDNA copies for each tissue were assayed in one 96-well plate (Applied Biosystem) together with non-template controls that did not contain cDNA and standards consisting of 10-fold serial dilutions of pCR-Blunt II TOPO® vector (Invitrogen, Carlsbad, USA) which contained the PCR product amplified by forward and reverse primer for the analyzed gene. For each reaction, 2 μl cDNA of each tissue was mixed with 25 μl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 1 μl of primer mix containing 20 μmol/L each of forward primer and reverse primer, 0.25 μl of 20 μmol/L Taqman probe, and 19.75 μl of diethyl pyrocarbonate (DEPC)-treated water (Nacalai tesque, Kyoto, Japan). All reactions were carried out by PCR amplification using a program of 40 cycles, each consisting of 95 °C for 5 min, 60 °C for 90 s. At the end of the reaction, the cycle threshold (C_t) values (i.e. the cycle numbers at

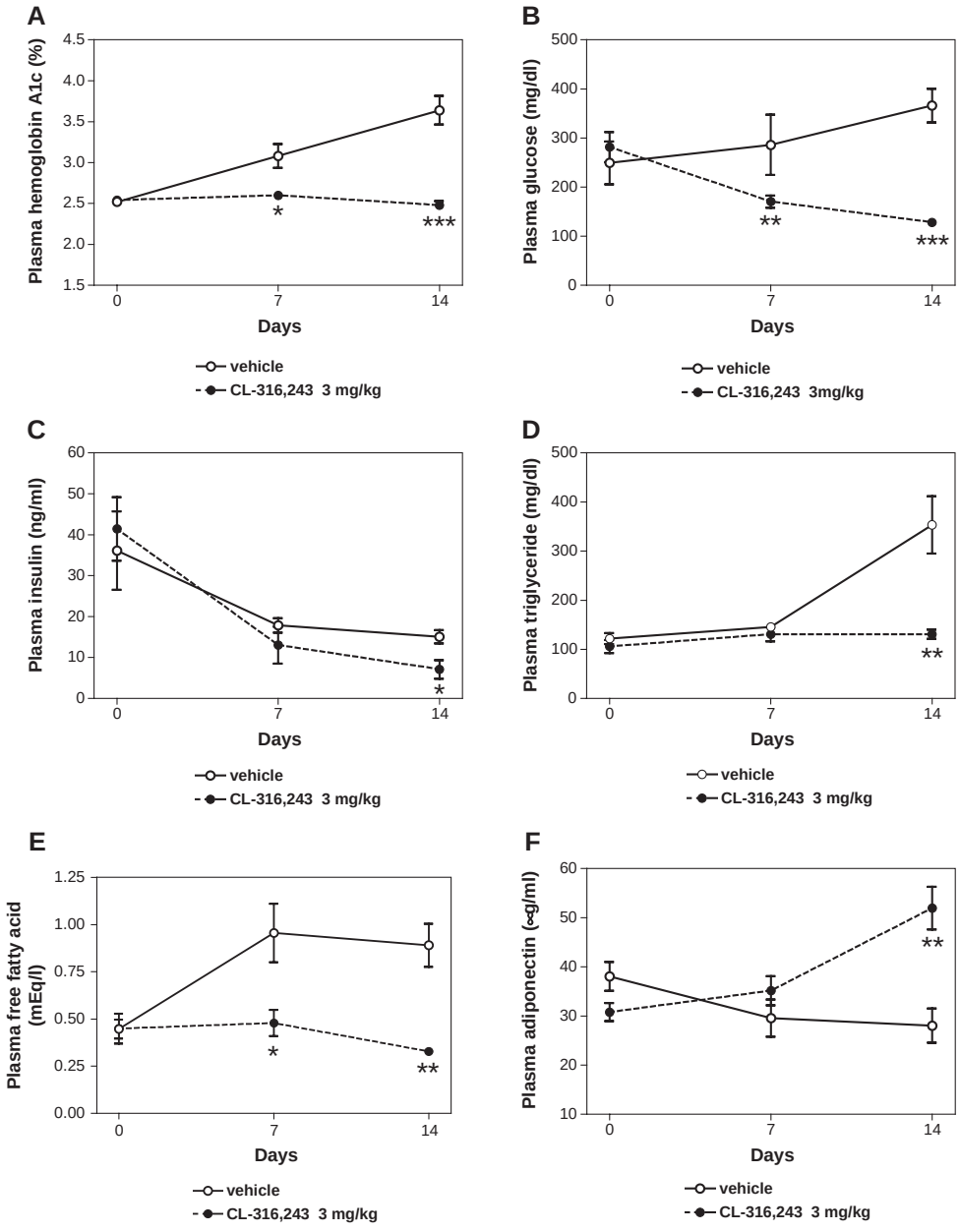

Fig. 2. Biochemical parameters in control and CL-316,243-treated *db*/*db* mice. A, plasma hemoglobin A1c; B, plasma glucose; C, plasma insulin; D, plasma triglyceride; E, plasma free fatty acid; F, plasma adiponectin. Mean ± S.E.M.; $n = 5$ in each group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. control group.

which fluorescent signals were obtained) were determined for standard and unknown samples. A standard curve was constructed by plotting the C_t values as a function of the log concentration of standard cDNA. On the basis of the C_t values of the unknown samples, their relative mRNA concentrations were determined and corrected using GAPDH mRNA as an internal standard.

2.5. Statistics

Statistical analysis of data was performed by means of an unpaired-*t* test (using Prism software from GraphPad Software, Inc., SanDiego, USA). Data are expressed as means ± S.E.M. Any *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Body weight, food intake and tissue weight

As shown in Fig. 1A, the CL-316,243-treatment group showed a smaller body weight gain than the control group. The food intake in the CL-316,243-treatment group was significantly lower than that of the control group only on day 1 (Fig. 1B). Total food intake over the whole 2 weeks' administration period was not significantly different between the two groups. The weight of epididymal white adipose tissue and brown adipose tissue in the CL-316,243-treatment group was less than that of the control group.

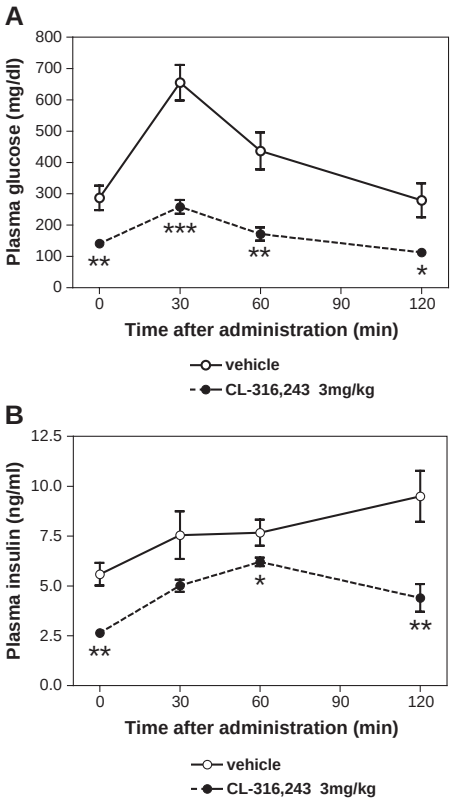

Fig. 3. Effect of CL-316,243 on results of an oral glucose tolerance test in *db*/*db* mice. The oral glucose tolerance test was performed after a 16-h fast. Blood samples were collected sequentially, then plasma levels of glucose and insulin were measured. A, plasma glucose; B, plasma insulin. Mean ± S.E.M.; $n = 5$ in each group. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control group.

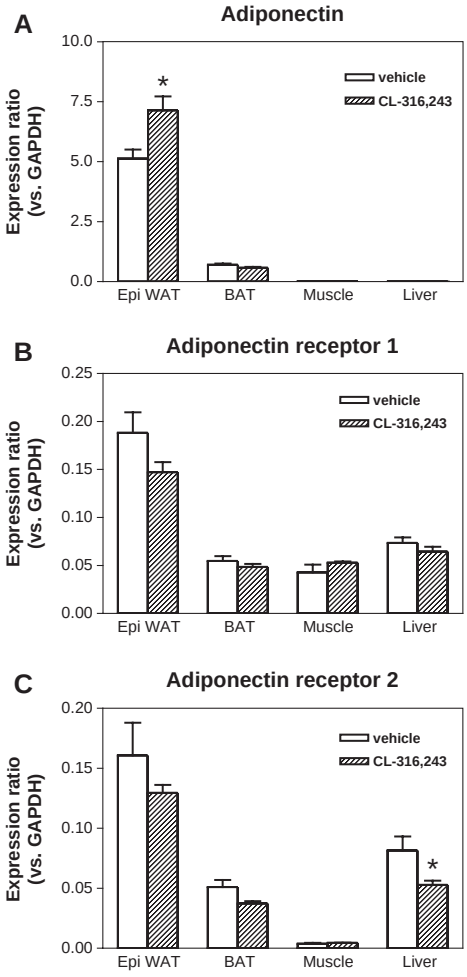

Fig. 4. Effects of 2 weeks' oral administration of CL-316,243 on adiponectin, adiponectin receptor 1, and adiponectin receptor 2 mRNA levels in epididymal white adipose tissue, brown adipose tissue, muscle and liver in *db*/*db* mice. Epi WAT is epididymal white adipose tissue, BAT is brown adipose tissue, GAPDH is glyceraldehyde-3-phosphate dehydrogenase. Mean ± S.E.M.; $n = 5$ in each group. $^*P < 0.05$ vs. control group.

3.2. Biochemical parameters

In the control group, there were time-dependent increases in plasma levels of hemoglobin A1c, plasma glucose and plasma free fatty acid but time-dependent decreases in plasma levels of insulin and adiponectin (Fig. 2).

In the CL-316,243-treatment group (versus the control group), there was a significant reduction in plasma levels of hemoglobin A1c, glucose and free fatty acids at both 1 and 2 weeks, and in plasma levels of insulin and triglyceride at 2 weeks, and a significant elevation in plasma levels of adiponectin at 2 weeks (Fig. 2).

3.3. Effects of CL-316,243 on oral glucose tolerance test

The oral glucose tolerance test performed on the first day after 2 weeks of administration of CL-316,243 elicited significantly lower plasma glucose levels at all times (0, 30, 60 and 120 min)

(Fig. 3A). The plasma insulin levels after glucose loading were significantly lower in the CL-316,243-treatment group than in the control group. These data indicate that this drug ameliorated insulin resistance.

3.4. Adiponectin, adiponectin receptor 1, and adiponectin receptor 2 mRNA expression

The expression of mRNA for adiponectin in epididymal white adipose tissue was significantly higher in the CL-316,243-treatment group (7.14 ± 0.58) than in the control group (5.13 ± 0.38) (Fig. 4A). In the other tissues examined, there was no significant difference in adiponectin mRNA expression between the two groups. Adiponectin receptor 1 mRNA expression was not significantly different between the CL-316,243-treatment group and the control group in any of the tissues tested. Adiponectin receptor 2 mRNA expression in the liver was significantly lower in the CL-316,243-treatment group (0.053 ± 0.003) than in the control group (0.081 ± 0.012) (Fig. 4C), but there was no such difference in any other tissues.

4. Discussion

In this study, we investigated the pharmacological effect of orally administered CL-3136,243 on adiponectin levels and adiponectin receptors in *db*/*db* mice. Administration of CL-316,243 for 2 weeks to these mice decreased the plasma levels of hemoglobin A1c, glucose, insulin, triglyceride and free fatty acid significantly (versus those of the control group). However, the plasma adiponectin level was significantly higher in the CL-316,243-treatment group than in the control group. The expression of adiponectin mRNA was up-regulated only in epididymal white adipose tissue following CL-316,243 treatment. Although we examined only a few important tissues, the possibility exists that this increase in plasma adiponectin level in the CL-316,243-treatment group was due to up-regulated expression in epididymal white adipose tissue only. A previous report showed that long-term administration of CL-316,243 increased the plasma adiponectin level in Brown Norway rats (Zhang et al., 2002). This is in accord with our result of an increased plasma adiponectin level. Moreover, pioglitazone, an agonist of peroxisome proliferator-activated receptor (PPAR) γ, may exert its antiatherosclerotic effect and ameliorate insulin resistance by increasing the serum adiponectin level (Maeda et al., 2001; Hirose et al., 2002). Adiponectin is an insulin-sensitive hormone that plays a central role in both glucose and lipid metabolism (Berg et al., 2002; Ukkola and Santaniemi, 2002; Haval, 2004). In the present study, CL-316,243 treatment ameliorated insulin resistance in the oral glucose tolerance test (by comparison with the control group). The improvement of metabolism of plasma free fatty acids and triglycerides in the CL-316,243-treatment group might play a part in this amelioration of insulin resistance. There is thus a possibility that the amelioration of insulin resistance was causally associated with the increase in the plasma adiponectin level.

Adiponectin receptor 1 and adiponectin receptor 2 have been cloned (Yamauchi et al., 2003), and adiponectin receptor 1 mRNA has been found to be highly expressed in human skeletal muscle (Debard et al., 2004; Civitarese et al., 2004). However, in the skeletal muscle of human subjects with type 2 diabetes, the expression of adiponectin receptor 1 is lower than in control subjects (Civitarese et al., 2004). Adiponectin receptor 2 is predominantly expressed in the liver (Yamauchi et al., 2003). In the present study, adiponectin receptor 1 mRNA was identified in epididymal white adipose tissue, interscapular brown adipose tissue, soleus muscle and liver, whereas adiponectin receptor 2 mRNA was identified in epididymal white adipose tissue, interscapular brown adipose tissue, liver and to a lesser extent, in soleus muscle. A recent report described adiponectin receptor 1 and adiponectin receptor 2 expression in porcine adipose tissue (Ding et al., 2004). Our results are the first to show the presence of adiponectin receptor 1 and adiponectin receptor 2 in white and brown adipose tissue in *db*/*db* mice. The possibility exists that, in *db*/*db* mice, epididymal white adipose tissue and brown adipose tissue both possess an autocrine system involving adiponectin. Although we detected no significant intergroup difference, there was a tendency for a decrease in adiponectin receptor 1 mRNA expression in epididymal white adipose tissue in the CL-316,243-treatment group, whereas the expression of mRNA for adiponectin receptor 2 in the liver was significantly lower in the CL-316,243-treatment group than in the control group. This is the first report that this β_3-adrenoceptor agonist decreases adiponectin receptor 2 expression in the liver of *db*/*db* mice. This decrease might be a result of the induced amelioration of lipid metabolism and insulin resistance. We suspect that the decrease in the expression of adiponectin receptor 2 mRNA may be related to a reduced need for adiponectin receptor 2 signaling in the liver in the CL-316,243-treatment group or that it may be due to a direct effect of the increased plasma adiponectin level in this group. To elucidate the interrelation between the amelioration of insulin resistance and adiponectin receptor 2 expression, further experiments will be needed.

In conclusion, we have shown that the β_3-adrenoceptor agonist CL-316,243 ameliorates insulin resistance in *db*/*db* mice with an accompanying increase in the plasma adiponectin level, an increased expression of adiponectin mRNA in epididymal white adipose tissue and a decreased expression of adiponectin receptor 2 mRNA specifically in the liver. These results suggest that the amelioration of insulin resistance seen in *db*/*db* mice treated with this β_3-adrenoceptor leads to a down-regulation of adiponectin receptor 2 mRNA expression specifically in the liver.

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