Effect of adipocyte β₃-adrenergic receptor activation on the type 2 diabetic MKR mice

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β₃-adrenergic receptors (β₃-AR) are primarily expressed in brown (BAT) and white adipose tissue (WAT) (3, 4, 11, 27). Their activation with selective agonists stimulates lipolysis and release of fatty acids in WAT and also activation of thermogenesis in BAT (1, 19, 28, 34). Reduction of murine β₃-AR number in adipose tissue of obese mice and Trp64Arg missense mutation of β₃-AR in obese humans both indicate a possible role of β₃-AR in obesity-related insulin resistance (12, 21, 38). However, the actual physiological role of β₃-AR in humans is not clear due to the lack of a highly selective β₃-AR agonist despite continuous trials over the past 20 years. Chronic administration of β₃-AR agonists have demonstrated antidiabetic and antiobesity effects in obese and diabetic rodent models (2, 20, 23, 29, 37, 41, 42, 44), yet the interaction between lipid and glucose metabolism induced by β₃-AR agonists treatment is not clear: there were inconsistent effects on lipid levels, glucose and/or insulin levels, and insulin sensitivity after chronic administration of β₃-AR agonists (1, 39). Possible antidiabetic effects of β₃-AR agonists have been suggested by the observation of improved insulin-stimulated glucose disposal with enhanced insulin action in adipose tissues but with unchanged circulating glucose, insulin, and lipid levels in normal Sprague-Dawley rats after chronic activation of β₃-AR (14).

We have recently developed a type 2 diabetic mouse model by overexpressing a dominant negative IGF-I receptor specifically in skeletal muscle (MKR mice) (16). Hybrid formation of the mutated IGF-IR with the endogenous IGF-I and insulin receptors caused impaired insulin and IGF-I receptor signaling pathways in skeletal muscle of the MKR mice. This defect in skeletal muscle resulted in hyperinsulinemia, dyslipidemia, and β-cell dysfunction leading eventually to type 2 diabetes, whereas total body fat and adipose tissue content were unchanged compared with normal wild-type (WT) mice. Whole body insulin resistance was associated with impaired insulin sensitivity in skeletal muscle as well as in adipose tissue and liver. A marked reduction of significantly elevated serum lipids [fatty acids (FA) and triglycerides (TG)] and liver lipid content following treatment with WY-14643, a peroxisome proliferator-activated receptor (PPAR)α agonist, was associated with improvement in insulin sensitivity and diabetes in MKR mice (24). However, rosiglitazone, a PPARγ agonist, failed to improve insulin resistance and diabetic status of MKR mice despite a significant reduction in circulating lipid levels (25). Rosiglitazone increased glucose uptake by adipose tissue but had no effect on insulin sensitivity in muscle or liver (25). This lack of effect of the thiazolidinediones was associated with adiponectin resistance seen in these mice and may be causally related.

In this study, to elucidate the effects of β₃-AR agonists in a nonobese diabetic mouse model, we treated MKR mice with a highly selective β₃-AR agonist, CL-316243. Chronic β₃-AR activation reversed the whole body insulin insensitivity with enhanced insulin action on liver and adipose tissue and reduced hyperinsulinemia and hyperglycemia in MKR mice, whereas insulin resistance in skeletal muscle was not affected. This improvement was possibly associated with the beneficial effect of β₃-AR agonist on the lipid profiles and lipid metabolism.

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MATERIALS AND METHODS

Animals. Generation and characterization of MKR mice have been described before (16). Homozygous MKR male mice (FVB/N background) used for the current study were subjected to Southern blot analysis for genotyping, as previously described (16). FVB/N male mice were used as controls. Mice were maintained on a 12:12-h light-dark cycle, and all experiments were performed in agreement with National Institutes of Health guidelines and with the approval of the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases.

Indirect calorimetry. Oxygen consumption and carbon dioxide production were measured using a four-chamber Oxymax system (Columbus Instruments, Columbus, OH) with one mouse/chamber and by testing transgenic mice simultaneously with littermate controls (17). Motor activity (total and ambulating) was determined by infrared beam interruption (Opto-Varimex mini, Columbus Instruments). Mice had free access to food and water. Resting O2 consumption was calculated as the average of the points with fewer than six ambulating beam breaks per minute and omission of the first hour of the experiment. The respiratory exchange ratio (RER; the ratio of CO2 produced to O2 consumed) was calculated using the same data points. Oxidation of carbohydrate produces an RER of 1.00, whereas FA oxidation results in an RER of 0.70 (32). Results for O2 consumption were normalized to (body weight)0.75 (40).

The effect of the β3-selective adrenergic agonist CL-316243 (6) on metabolic rate was measured as follows with each mouse serving as its own control. At ~9:00 AM, mice were placed into the calorimetry chambers (prewarmed to 30°C), and baseline data were collected. CL-316243 was injected (1 mg/kg ip) 3 h later, and after a 1-h delay data were collected for 3 h.

Acute administration of CL-316243. Six- to seven-week-old male WT and MKR mice were injected intraperitoneally either with CL-316243 (1 mg·kg−1·day−1) or with an equivalent volume of sterile saline. After 20 min, blood was collected from tail vein for serum FA levels.

Chronic administration of CL-316243. Six- to seven-week-old male WT and MKR mice were injected intraperitoneally with CL-316243 (1 mg·kg body wt−1·day−1) or with an equivalent volume of sterile saline daily (10:00 AM) for 3 wk. Body weight and food intake were measured daily or weekly, respectively. Blood was collected from the tail vein weekly between 11:00 AM and noon in nonfasting state. A glucometer (One Touch II, LifeScan) was used to measure glucose levels in the nonfasting state. Oxygen consumption was measured on day 20 for 24 h at 23°C. Mice were euthanized on day 21 of treatment after anesthetization using 2.5% Avertin (15–17 mg/g body wt) in the nonfasting state between 11:00 AM and noon. Tissues were collected for FA oxidation assay and immediately frozen in liquid nitrogen for measurement of tissue TG levels and RNA analysis.

Serum analysis. Serum was obtained from the tail vein between 11:00 AM and noon in the nonfasting state. Serum FA and TG levels were measured using a FA assay kit (Roche, Indianapolis, IN) and GPO-Trinder kit (Sigma, St. Louis, MO), respectively. Serum insulin, leptin, and adiponectin levels were determined using radioimmunoassay (Linco Research, St. Charles, MO).

Insulin and glucose tolerance tests. Insulin and glucose tolerance tests were done after 5–7 h of fasting. Mice were injected intraperitoneally with either insulin (0.5 U/kg body wt) or glucose (2 g/kg body wt). Blood glucose levels were determined from the tail vein at 0, 30, and 60 min after insulin injection and at 0, 15, 30, 60, and 120 min after glucose injection.

Hyperinsulinemic euglycemic clamp. The clamp studies were performed as developed by Kim et al. (26). Mice were treated with either CL-316243 or saline for 2 wk, as described above. On day 14 of treatment (4 days before the clamp experiment), mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. A catheter was inserted into a lateral incision on the right side of the neck and advanced into the superior vena cava via the right internal jugular vein. The catheter was then sutured into place according to the protocol of MacLeod and Shapiro (30). The day before the clamp analysis, mice received the final injection of CL-316243 or saline at 11:00 AM and were then fasted overnight. To conduct experiments in conscious animals with minimal stress, a tail restraint method was used during experiments. The basal rates of glucose turnover were measured by continuous infusion of [3-3H]glucose (0.02 µCi/min) for 120 min, which followed a bolus of 2.5 µCi, starting at 9:00 AM. Blood samples (20 µl) were taken at 90 and 115 min of the basal period for the determination of plasma [3H]glucose concentration. A 120-min hyperinsulinemic euglycemic clamp was started at 11:00 AM. Insulin was infused as a bolus of 150 mU/kg over a period of 3 min followed by continuous insulin infusion at the rate of 2.5 mU·kg−1·min−1 (Humulin R; Eli Lilly, Indianapolis, IN) to raise the plasma insulin concentration to ~2 ng/ml. During the clamp study, mice were restrained, and blood samples (20 µl) were collected via a small nick in the tail vein at 15-min intervals for the immediate measurement of plasma glucose concentration, and 200 µl of blood was infused at variable rates to maintain plasma glucose at ~160 mg/dl in WT mice (control MKR mice were clamped at ~240 mg/dl; because of severe insulin resistance, this was the lowest glucose level we could reach). Insulin-stimulated whole body glucose flux was estimated using a primed continuous infusion of high-pressure liquid chromatography-purified [3-3H]glucose (10-µCi bolus, 0.1 µCi/min; NEN Life Science Products, Boston, MA) throughout the clamps. To estimate insulin-stimulated glucose transport activity and metabolism in skeletal muscle, 2-deoxy-o-[1-14C]glucose (NEN Life Science Products) was administered as a bolus (10 µCi) at 45 min before the end of clamps. Blood samples (20 µl) were taken at 80, 85, 90, 100, 110, and 120 min after the start of clamps for the determination of plasma [3H]glucose, 2-deoxy-o-[1-14C]glucose, and [1-14C]O2 concentrations. Additional blood samples (10 µl) were collected before the start and at the end of clamp studies for measurements of plasma insulin concentration. All infusions were performed using microdialysis pumps (CMA/Microdialysis, Acton, MA). At the end of the clamp period, animals were anesthetized with ketamine-xylazine injection.

Within 5 min, gastrocnemius muscle from hindlimbs, epididymal and brown adipose tissue, and liver were removed. Each tissue, once exposed, was dissected within 2 s, frozen immediately using liquid nitrogen-cooled aluminum blocks, and stored at −70°C for later analysis.

Tissue TG content determination. Livers were powdered, and tissue TG were extracted in chloroform-methanol solution. The solution was centrifuged after addition of 2% KH2PO4, and the lower phase was collected for evaporation. Then, isopropyl alcohol was added to dissolve the remaining pellet. The amount of released glycerol was measured by radiometric assay as described before (7, 9).

Body fat content determination. Body composition was measured in nonanesthetized mice by use of the Bruker minispec NMR analyzer mq10 (Bruker Optics, Woodlands, TX).

Food intake. Mice were caged individually and treated with either CL-316243 or vehicle (sterile saline), as described above. The amount of food in the feeding container was measured at days 0 and 10 of treatment. Food intake was normalized to the body weight of each mouse and was expressed as grams of food per gram of body weight0.75 per day.

TG secretion. TG secretion was determined by the measurement of the increased circulating TG after lipase inhibition by WR-1339 (Sigma), as described previously (13). At day 21 of treatment with either CL-316243 or saline, mice were given ad libitum access to a fat-free diet (Frosted Flakes; Kellogg, Battle Creek, MI) for 4 h. Mice were then anesthetized with ketamine (100 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg; Phoenix Scientific, St. Joseph, MO). WR-1339 (100 µl from a solution of 1:10 dilution in phosphate-buffered saline) was injected into the tail vein.
and blood samples were collected at 0, 1, and 2 h. Results were expressed as milligrams of TG per kilogram body weight per hour, and plasma volume was presumed as a 3.5% of body weight.

**TG clearance test.** TG clearance from the circulation in 4-h-fasted mice was measured after gavage of 400 μl of peanut oil after 3 wk of treatment. Blood samples were withdrawn hourly from the tail vein for 6 h.

**FA oxidation assay.** Isolated free adipocytes from epididymal adipose tissue (36) of mice, after 3 wk of treatment, were incubated with BSA-bound palmitic acid (4.3 μmol/l) and [1-14C]palmitic acid [9,10-3H(N), 55.6 pmol/l; PerkinElmer Life Sciences, Boston, MA] in 5 mM glucose, Krebs-Ringer-HEPES-albumin buffer (pH 7.4, 20 mg/ml FA-free BSA) buffer at 37°C for 0, 30, and 60 min, respectively. At every time point, the cell suspension was immediately added into a microtube, which contained the same volume of mineral oil, and was then briefly centrifuged. The lower phase was added into a column filled with 1 ml of resin (Bio-Rad AG1-X8, 200–400 mesh), which retains FAs. The column was washed with water, and the eluate was counted for 3H2O production. Adipocyte DNA content was measured by fluorometric quantification assay (15) using bis-benz-amide as a dye and calf thymus high polymerized DNA (Sigma) as a standard. The results were expressed as the value of oxidized palmitic acid (pmol) normalized by adipocyte DNA (μg). The stock solutions of palmitic acid bound to FA-free BSA were prepared as described previously (8), and the nonesterified fatty acid concentration was verified using a NEFA C kit (Wako Chemicals, Richmond, VA).

**FA oxidation on isolated soleus muscle.** All experiments were performed in Krebs-Henseleit buffer (KHB; in mM: 118.5 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 0.5 EDTA, and 25 NaHCO3, supplemented with 5 mM d-glucose) (22). All BSA and palmitate-BSA complexes used were dialyzed extensively against NaHCO3, supplemented with 5 mM D-glucose) (22). All BSA and nonesterified fatty acid concentration was measured by fluorometric quantification assay (15) using bis-benz-amide as a dye and calf thymus high polymerized DNA (Sigma) as a standard. The results were expressed as the value of oxidized palmitic acid (pmol) normalized by adipocyte DNA (μg). The stock solutions of palmitic acid bound to FA-free BSA were prepared as described previously (8), and the nonesterified fatty acid concentration was verified using a NEFA C kit (Wako Chemicals, Richmond, VA).

**RNA analysis.** Total RNA from liver, WAT, and BAT was isolated using TRiZol reagent (Life Technologies, Rockville, MD), and Northern blot analysis was performed as previously described. (23)

**Histology.** The left gonadal fat pad was removed following euthanasia and fixed overnight in 4% paraformaldehyde in PBS. The tissues were then transferred to 70% ethanol and embedded in paraffin. Samples were cut into 5-μm sections, and hematoxylin-eosin staining was performed. Immunostaining for cytochrome-c oxidase was performed using an antibody from Molecular Probes (Eugene, OR).

**Statistical analysis.** All data are expressed as means ± SE. Student’s t-test (unpaired and paired) was used to determine statistically significant differences between genotype.

**RESULTS**

**Acute effect of CL-316243 on MKR mice.** To determine whether β3-adrenergic responsiveness is intact in MKR mice, we measured serum FAs and metabolic rate in WT and MKR mice after acute β3-adrenergic stimulation. Serum FAs rose four- to sixfold in both WT and MKR mice, indicating an increase in the lipolysis from BAT and WAT (Fig. 1A). In response to acute CL-316243 treatment, both WT and MKR mice increased O2 consumption by ∼100% and decreased the RER (ratio of CO2 produced to O2 consumed), an indicator of metabolic fuel source, suggesting increased net FA oxidation. Therefore, these data demonstrate that intact β3-adrenergic responses are preserved in MKR mice.

**Effect of chronic CL-316243 treatments on body composition.** To explore potential antidiabetic effects of β3-adrenergic stimulation in MKR mice, we treated mice with CL-316243 for 3 wk; there was no effect on body weight. However, total fat content decreased significantly in both WT and MKR mice (25 and 16%, respectively; Table 1). Interestingly, various fat depots were affected differently: epididymal pad weight was decreased by 33 and 50% in WT and MKR mice, respectively; however, inguinal fat weight did not change after 3 wk of treatment with CL-316243 (Table 1). BAT did not change after CL-316243 treatment in WT mice [194 ± 27 vs. 140 ± 15 mg, *P* = not significant (NS)] or in MKR mice (120 ± 3 vs.138 ± 11 mg, *P* = NS).

**Materials and Methods.** Results are expressed as means ± SE; *n* = 6–8/group. *Differences between treated and untreated group were significant at *P* < 0.05.
As expected, chronic CL-316243 treatment caused significant increases in O₂ consumption. Average 24-h O₂ consumption measured at ambient temperature (23°C) increased by 12 and 11% in MKR and WT mice, respectively (Table 1). This increase in O₂ consumption was correlated with a slight, probably compensatory, increase in food intake of both WT and MKR mice (14 and 10%, respectively; Table 1). This may explain why both WT and MKR mice maintained body weight and showed only modest reductions in total fat mass despite a chronic increase in metabolic rate.

Treatment caused a reduction in the serum leptin levels by 25 and 15% in WT and MKR mice, presumably due to decreased body fat content (Table 1). Serum adiponectin level in WT mice was significantly increased, but not in MKR mice, in response to CL-316243 treatment (Table 1).

### Antidiabetic effect of chronic CL-316243 treatment on MKR mice.

3-Adrenergic agonist CL-316243 treatment of MKR mice for 3 wk caused a marked improvement in hyperglycemia and hyperinsulinemia. Blood glucose levels fell from 242 ± 28.4 to 125 ± 3.1 mg/dl (Fig. 2A), whereas serum insulin levels fell from the hyperinsulinemic range of 21 ± 3.3 to 4 ± 3.1 μU/ml (Fig. 2B). This improvement was accompanied by a significant decrease in area under the curve for glucose tolerance tests (Fig. 2C, D, E) in MKR mice compared to WT mice. The liver weight was significantly reduced in MKR mice treated with CL-316243 compared to untreated MKR mice (Table 1). In conclusion, chronic CL-316243 treatment improved metabolic parameters in MKR mice, suggesting a potential therapeutic effect of this 3-AR agonist in diabetes.

### Table 1. Oxygen consumption and total body fat content

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + CL</th>
<th>MKR</th>
<th>MKR + CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>28.5 ± 0.4</td>
<td>27.4 ± 1.1</td>
<td>23.9 ± 0.5†</td>
<td>24.2 ± 0.5</td>
</tr>
<tr>
<td>Food intake, g</td>
<td>0.35 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.39 ± 0.0</td>
<td>0.43 ± 0.1</td>
</tr>
<tr>
<td>% Fat content (total body fat content/body wt)</td>
<td>1.06 ± 0.1</td>
<td>0.71 ± 0.1*</td>
<td>0.98 ± 0.0</td>
<td>0.49 ± 0.1*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.42 ± 0.0</td>
<td>1.4 ± 0.1</td>
<td>1.51 ± 0.1</td>
<td>1.48 ± 0.1</td>
</tr>
<tr>
<td>Liver weight, % total body wt</td>
<td>0.89 ± 0.1</td>
<td>0.97 ± 0.2</td>
<td>0.74 ± 0.0</td>
<td>0.83 ± 0.0</td>
</tr>
<tr>
<td>Serum leptin, ng/ml</td>
<td>3.89 ± 0.6</td>
<td>2.93 ± 0.4</td>
<td>3.87 ± 0.3</td>
<td>3.29 ± 0.5</td>
</tr>
<tr>
<td>Serum adiponectin, μg/ml</td>
<td>2.73 ± 0.9</td>
<td>5.08 ± 0.7*</td>
<td>6.64 ± 0.9†</td>
<td>6.62 ± 1.1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 8–9/group. Six- to seven-week-old MKR and wild-type (WT) mice were injected with saline or CL-316243 (CL; 1 mg/kg ip) for 3 wk. In the fed state, O₂ consumption was measured on day 20 for 24 h at 23°C, and serum and tissues were collected. Total body fat content was measured in awake mice. *P < 0.05 treated vs. untreated group; †P < 0.05 treated vs. WT.

As expected, chronic CL-316243 treatment caused significant increases in O₂ consumption. Average 24-h O₂ consumption measured at ambient temperature (23°C) increased by 12 and 11% in MKR and WT mice, respectively (Table 1). This increase in O₂ consumption was correlated with a slight, probably compensatory, increase in food intake of both WT and MKR mice (14 and 10%, respectively; Table 1). This may explain why both WT and MKR mice maintained body weight and showed only modest reductions in total fat mass despite a chronic increase in metabolic rate.

Treatment caused a reduction in the serum leptin levels by 25 and 15% in WT and MKR mice, presumably due to decreased body fat content (Table 1). Serum adiponectin level in WT mice was significantly increased, but not in MKR mice, in response to CL-316243 treatment (Table 1).
2.4 ng/ml (Fig. 2B). In contrast, chronic CL-316243 treatments did not alter the levels of these parameters in WT mice.

Insulin tolerance tests were performed after 3 wk of CL-316243 treatment. Consistent with previous studies (16, 24, 25), MKR mice exhibited severe insulin insensitivity compared with WT mice (Fig. 2C). Chronic CL-316243 treatment in MKR mice significantly enhanced the ability of insulin to clear blood glucose at 60 min from 88 to 63% of initial levels (to the levels of WT mice), indicating enhanced insulin sensitivity (Fig. 2C). A slight increase in insulin sensitivity was observed in WT mice after CL-316243 treatment (Fig. 2C).

Consistent with our earlier studies (16, 25), control MKR mice had higher basal fasting glucose levels compared with WT mice and were glucose intolerant (Fig. 2D). Glucose tolerance was markedly improved in MKR mice after treatment (Fig. 2D). The area under curve (AUC) was significantly reduced by 63% in MKR mice after CL-316243 treatment compared with saline-treated MKR mice (Fig. 2E). CL-316243 treatment caused a significant decrease in AUC of WT mice by 20% (Fig. 2E).

Taken together, these data demonstrate that chronic β3-adrenergic agonist treatment significantly improved insulin sensitivity and glucose homeostasis in MKR mice.

**CL-316243 treatment increased insulin sensitivity in hepatic and adipose tissue in MKR mice.** MKR mice exhibit insulin resistance in all major insulin responsive tissues: muscle, liver, and fat (16). To assess the mechanism of the insulin-sensitizing effect of CL-316243, we performed hyperinsulinemic euglycemic clamp studies. After an overnight fast, CL-316243-treated MKR mice showed a significant reduction in basal serum insulin levels to the level of WT mice (from 2.4 ± 1.3 to 0.5 ± 0.1 ng/ml) despite unchanged hyperglycemia. The glucose infusion rate required to maintain euglycemia was significantly higher after CL-316243 treatment in both MKR and WT mice, suggesting improvement in whole body insulin sensitivity. In MKR mice, basal endogenous glucose production (EGP) was significantly higher than in WT controls; basal serum insulin, 178 ± 11 vs. 184 ± 11 ng/ml; basal EGP, 0.5 ± 0.1 vs. 0.5 ± 0.1 μmol/kg body wt \(^{-1}\) min \(^{-1}\); Clamp serum glucose, 174 ± 7 vs. 160 ± 6 mg/dl; Clamp serum insulin, 1.8 ± 0.4 vs. 1.6 ± 0.2 μmol/kg body wt \(^{-1}\) min \(^{-1}\); Whole body glycogen synthesis, 127 ± 25 vs. 134 ± 22 μmol/kg body wt \(^{-1}\) min \(^{-1}\) in MKR mice after treatment (Fig. 3C). Liver glycogen synthesis rate, g, was reduced by 63% in MKR mice after CL-316243 treatment (Fig. 4E). CL-316243 treatment reduced serum TG level in MKR mice compared with WT mice. Certain limitations in the hyperinsulinemic euglycemic clamp technique may explain the data. These observations are probably due to significantly increased glucose levels during clamps in MKR mice compared with WT mice. This increased glucose uptake is most likely independent of the insulin-stimulated pathway in MKR mice.

**Chronic CL-316243 treatment improves lipid metabolism in MKR mice.** We next studied the effects of chronic CL-316243 treatment on lipid metabolism in WT and MKR mice. Treating MKR mice with CL-316243 for 3 wk significantly lowered serum and hepatic lipid levels. Serum TG levels fell from the hypertriglyceridemic range of 148 ± 14.7 to 60 ± 21.3 mg/dl in MKR mice after treatment (Fig. 4A), whereas serum FA levels were reduced from 0.4 ± 0.03 to 0.2 ± 0.03 mg/ml in WT mice after treatment (Fig. 4B). CL-316243 treatment significantly decreased liver TG content by ~38% in MKR mice (Fig. 4C). The decreased liver TG levels correlated well with a reduction in in vivo TG secretion rate (by ~37%) in MKR mice after treatment (Fig. 4D). However, treatment did not change circulating and liver TG and FA levels in WT mice (Fig. 4E). The expressions in liver of fatty acid synthase (FAS), steroyl-CoA desaturase (SCD)-1, glycogen synthase, glucokinase (GK), phosphoenolpyruvate carboxykinase (PEPCK), and PPARα mRNA remained unchanged in both genotypes after treatment (data not shown).

To determine whether the reduction of serum TG level in MKR mice after CL-316243 treatment is associated with enhanced TG clearance, we measured serum TG levels after an oral lipid load. At 3 h, serum TG peaked at 677 and 805 mg/dl in WT and MKR mice, respectively, and then fell gradually (Fig. 4E). CL-316243 treatment lowered the peak of serum TG levels to 184 mg/dl in MKR mice and 459 mg/dl in WT mice 2 h after the lipid load, and serum TG were reduced rapidly.

### Table 2. Effects of CL-316243 treatment on metabolic parameters during basal and hyperinsulinemic euglycemic clamp

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>WT + CL</th>
<th>MKR</th>
<th>MKR + CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>25.3 ± 0.4</td>
<td>24.9 ± 0.8</td>
<td>20.6 ± 0.9†</td>
<td>21.6 ± 0.7†</td>
</tr>
<tr>
<td>Basal serum glucose, mg/dl</td>
<td>178 ± 11</td>
<td>184 ± 11</td>
<td>344 ± 25†</td>
<td>330 ± 45†</td>
</tr>
<tr>
<td>Basal EGP, μmol/kg body wt (^{-1}) min (^{-1})</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>2.4 ± 1.3†</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>Clamp serum glucose, mg/dl</td>
<td>174 ± 7</td>
<td>160 ± 6</td>
<td>242 ± 22†</td>
<td>181 ± 8*</td>
</tr>
<tr>
<td>Clamp serum insulin, μmol/kg body wt (^{-1}) min (^{-1})</td>
<td>1.8 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>5.6 ± 2.5†</td>
<td>1.2 ± 0.1*</td>
</tr>
<tr>
<td>Whole body glycogen synthesis, μmol/kg body wt (^{-1}) min (^{-1})</td>
<td>127 ± 25</td>
<td>134 ± 22</td>
<td>144 ± 24</td>
<td>62 ± 10*</td>
</tr>
<tr>
<td>Liver glycogen synthesis rate, g</td>
<td>5.0 ± 2.3</td>
<td>12.6 ± 3.4</td>
<td>1.3 ± 0.2</td>
<td>6.4 ± 2.4*</td>
</tr>
<tr>
<td>Muscle (QC) glycogen synthesis rate, g</td>
<td>2.8 ± 0.5</td>
<td>4.1 ± 1.2</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.7</td>
</tr>
</tbody>
</table>
indicating more efficient TG clearance in MKR mice after treatment (Fig. 4E). This was not due to poor TG uptake from the intestine, because, in the absence of TG clearance [caused by WR-1339, a lipoprotein lipase (LPL) inhibitor], serum TG increased equally in control and CL-316243-treated mice after oral TG load (Fig. 4F). Taken together, these data indicate that CL-316243 treatment accelerated TG clearance in both WT and MKR mice.

Chronic CL-316243 treatment increases adipose tissue FA oxidation in MKR mice. It is presumed that β3-adrenergic stimulation increases muscle (2) and adipose tissue FA oxidation, thus leading to rapid reduction of circulating TG levels and hepatic TG content. The level of muscle FA oxidation was significantly decreased by 39% in MKR mice after treatment but was not altered in WT mice after treatment (Fig. 5A). We next measured FA oxidation in the isolated epididymal adipose tissue following chronic treatment. CL-316243 treatment caused a marked increase (fourfold) in adipose tissue FA oxidation in MKR mice, but only a onefold increase in WT mice (Fig. 5B). LPL mRNA levels showed a tendency to increase in WAT in both WT (1.63 ± 0.01 vs. 3.72 ± 0.46 AU) and MKR (5.06 ± 1.2 vs. 6.33 ± 0.88 AU) mice, whereas in BAT it tended to increase only in MKR mice (2.62 ± 1.36 vs. 4.05 ± 1 AU). Therefore, these data suggest that adipose tissue, rather than muscle, is primarily involved in the reduced lipid levels in MKR mice after chronic β3-adrenergic agonist treatment.

Several studies have reported that β3-adrenergic agonists decrease the size of adipocytes in epididymal adipose tissue (10, 18, 20). Concomitant changes in mitochondrial content and cytochrome-c oxidase expression in epididymal or inguinal adipose tissue have also been reported (23). Hematoxylin-eosin staining in epididymal adipose tissue showed typical unilocular cells in both WT and MKR mice (Fig. 5C, left). As expected, treatment with CL-316243 caused the formation of small adipocytes and multilocular adipocytes in epididymal adipose tissues of MKR mice (Fig. 5C, right). Accordingly, immunostaining for cytochrome-c oxidase showed an increase in both WT and MKR epididymal adipose tissue (Fig. 5D). Compared with MKR mice, the effects of CL-316243 on morphology changes and cytochrome-c oxidase content in epididymal adipose tissue observed in WT mice after treatment were less marked (Fig. 5, C and D, left).

DISCUSSION

The present study demonstrated that chronic treatment with a β3-adrenergic agonist, such as CL-316243, markedly improved hyperglycemia and hyperinsulinemia concurrently with significantly improved lipid profiles (circulating and tissue lipid contents) and metabolism in MKR mice that are diabetic but not obese. This improvement was paralleled by enhanced whole body insulin sensitivity, which was associated with increased hepatic insulin responsiveness and insulin-stimulated glucose uptake in adipose tissue. These results suggest that potentiation of insulin action on liver and adipose tissues plays a crucial role in the antidiabetic effects of β3-adrenergic
agonists in MKR mice that are genetically defective in muscle insulin action.

The importance of hepatic insulin action for whole body insulin sensitivity and glucose homeostasis in MKR mice has been shown previously (24, 25). An increase in hepatic lipo- 

3-AR mRNA, was associated with hepatic insulin resistance 

(25). Enhanced hepatic insulin action was correlated with a reduction in hepatic TG levels after treatment of WY-14643, a 

PPARα agonist (24). Consistently, reduction in hepatic TG 

levels, paralleled with a decrease in TG secretion rate, was related to the improvement in hepatic insulin sensitivity in MKR mice after chronic CL-316243 treatment. This improved hepatic insulin sensitivity was shown by reduced EGP and increased hepatic glycogen synthesis rate in response to insulin during the hyperinsulinemic euglycemic clamp. Due to exclu-

sive and abundant expression of 3-AR in adipose tissue, this 

effect of CL-316243 on hepatic insulin sensitivity could be secondary to the increased fatty acid utilization in adipose tissues, resulting in reduced hepatic lipid contents. In fact, CL-316243 treatment did not affect the expression of hepatic mRNA levels such as FAS, SCD-1, glycogen synthase, and PEPCK mRNA (data not shown).

The mechanisms responsible for enhanced insulin sensitivity in adipose tissues after activation of 3-ARs include the appearance of small adipocytes, increased expression of uncoupling protein-1 in both BAT and WAT (23, 35), the expression of insulin receptors and GLUT4, increased postreceptor activity, increased mitochondrial content (23), cytochrome-c oxidase content (23), and impaired adipogenesis by downregulation of PPARγ and aP2 gene (23, 31). Chronic treatment with the 3-adrenergic agonist in MKR mice caused an appearance of small adipocytes in both inguinal and epididymal adipose tissues, whereas there was a lesser effect in WT mice. Furthermore, the 3-adrenergic agonist induced an improvement in insulin sensitivity in adipose tissues as well as in liver, leading to an increase in whole body insulin sensitivity and glucose metabolism in MKR mice. Improved insulin action in adipose tissues, but not in skeletal muscle, was responsible for the enhanced whole body insulin-stimulated glucose disposal in nonobese rats after 3-AR activation (14). These results indicate that adipose tissue is a possible primary target site for the antidiabetic effect of the 3-adrenergic agonist.

The expression levels of 3-AR are adipose tissue depot dependent. For example, in humans, adipocytes derived from intra-abdominal or visceral depots reveal more 3-AR with better responsiveness to 3-adrenergic agonists, whereas adipocytes derived from subcutaneous depots have few 3-ARs. Consistently, there was a greater reduction in epididymal adipose tissue weight than in inguinal adipose tissue weight in both WT and MKR mice after CL-316243 treatments. This reduction of epididymal adipose tissue weight as well as total
body fat content in both WT and MKR mice indicate a similar antiobesity effectiveness of \( \beta_3 \)-adrenergic agonist on both WT and MKR mice. It is presumed that enhanced expression level of LPL and appearance of lipid in BAT result in increased TG clearance with chronic activation of \( \beta_3 \)-AR (10, 18, 20, 33). Chronic administration of CL-316243 in MKR mice markedly enhanced TG clearance, whereas there was a lesser effect in WT mice. Consistently, morphological changes in epididymal adipose tissue such as formation of multilocular adipocytes, a possible indicator of reappearance of BAT, were less obvious in WT mice after treatment compared with treated MKR mice. Therefore, the effectiveness of \( \beta_3 \)-adrenergic agonist on WAT by appearance of multilocular adipocytes, in part, contribute to the increase in TG clearance.

Stimulation of FA oxidation as well as increased lipolysis in WAT is associated with the reduction in circulating FA levels after chronic activation of \( \beta_3 \)-AR (5, 31, 43). Our findings demonstrated increased lipolysis in an acute response to CL-316243 treatments and enhanced FA oxidation in adipocytes of MKR mice after chronic CL-316243 treatment. However, FA oxidation in gastrocnemius muscle was not increased in MKR mice after chronic CL-316243 treatment, possibly related to less availability of FAs by a reduction of circulating FA levels after chronic activation of \( \beta_3 \)-AR and exclusively high expression of \( \beta_3 \)-AR in adipose tissues. It has been reported that muscle LPL activity was not affected with activation of \( \beta_3 \)-AR (33). These data indicate the significant role of adipose tissue in the reduction of circulating FA levels after chronic activation of \( \beta_3 \)-AR.

In summary, intact \( \beta_3 \)-adrenergic responses on \( \beta_3 \)-adrenergic activation with CL-316243 treatment are retained in diabetic MKR mice that have impaired insulin and IGF-I signaling pathways in skeletal muscle. Chronic administration of CL-316243 significantly improves whole body insulin sensitivity and glucose homeostasis in MKR mice, mainly due to enhanced insulin action on liver and adipose tissues. This enhanced insulin sensitivity was associated with the beneficial effect of the \( \beta_3 \)-adrenergic agonist CL-316243 on lipid metabolism in MKR mice. Reduction in circulating and hepatic lipid levels was, in part, related to increased TG clearance, decreased TG secretion rate, and increased adipocyte FA oxidation in MKR mice in response to CL-316243 treatment. Increased metabolic rate paralleled the reduction in epididymal adipose tissue and total body fat content in MKR mice after CL-316243 treatment.

In conclusion, enhanced insulin action on liver and adipose tissue, mainly due to increased adipocyte FA oxidation, with \( \beta_3 \)-AR activation is able to counterbalance the diabetes state of MKR mice that are genetically defective in IGF-I/insulin receptor signaling pathways of skeletal muscle.

![Fig. 5. Effects of CL-316243 on FA oxidation and morphology of adipose tissues. A: FA oxidation by soleus muscle. Palmitic acid (PA) oxidation rates in isolated soleus muscle were determined at an FA-to-BSA ratio of 0.67 (n = 6–8/group). B: palmitic acid oxidation by epididymal adipose tissues. Isolated adipocytes were incubated with BSA-bound palmitic acid (4.33 \( \mu \)mol/l) for 0, 30, and 60 min (n = 3/group). FA oxidation was measured as described in METHODS AND MATERIALS. C: morphology of epididymal adipose tissue from hematoxylin-eosin staining. D: immunostaining for presence of cytochrome-c oxidase in both WT and MKR epididymal adipose tissue before and after treatment with CL-316243. Six- to seven-week-old MKR and WT mice were injected with saline (■) or CL-316243 (○) (1 mg/kg ip) for 3 wk. Muscle and epididymal adipose tissue were removed to measure FA oxidation or to process morphology study in the nonfasting state. Data are expressed as means ± SE. *Differences between treated and untreated were significant at \( P < 0.05 \).](http://ajpendo.physiology.org/.../E1234.png)
GRANTS
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REFERENCES
29. Virtanen KA, Rouru J, Hanninen V, Savontaus E, Rovani T, Teirmann T, Koulum H, and Huupponen R. Chronic treatment with BRL...


