Amelioration by KRP-297, a New Thiazolidinedione, of Impaired Glucose Uptake in Skeletal Muscle From Obese Insulin-Resistant Animals

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We examined the effect of KRP-297, a new thiazolidinedione derivative, on glucose uptake in the soleus muscle of two animal models of insulin resistance that show moderate (ob/ob mice) and severe (db/db mice) hyperglycemia. Insulin-stimulated 2-deoxyglucose (2DG) uptake in soleus muscle was 53.8% lower in ob/ob mice versus lean mice ($P < .05$). When administered to ob/ob mice, KRP-297 (0.3 to 10 mg/kg) decreased plasma glucose and insulin levels and improved the impaired insulin-stimulated 2DG uptake in soleus muscle in a dose-dependent manner. Soleus muscle from db/db mice exhibited defects in both basal (35.0% decrease, $P < .01$) and insulin-stimulated (50.5% decrease, $P < .01$) 2DG uptake. These defects were improved by treatment with KRP-297 (0.3 to 10 mg/kg). Moreover, KRP-297 prevented severe hyperglycemia and the marked decrease in pancreatic insulin content in db/db mice. These results suggest that KRP-297 treatment is useful to prevent the development of diabetic syndromes in addition to ameliorating the impaired glucose transport in skeletal muscle.

NON–INSULIN-DEPENDENT diabetes mellitus (NIDDM) is associated with a marked impairment in the ability of insulin to stimulate glucose uptake in skeletal muscle, a major site of glucose disposal in vivo.[1] This defect is manifested in vivo as a reduced insulin-stimulated glucose disposal rate detected by the euglycemic clamp technique. Nevertheless, a study using perfused hindquarters or isolated soleus muscle also suggested an impairment in basal (without insulin) glucose uptake in genetically insulin-resistant animals such as obese (ob/ob) mice[2] and obese-diabetic (db/db) mice.[3] These mice are widely used as animal models of insulin resistance with obesity.[4] Ob/ob mice exhibit moderate hyperglycemia and marked hyperinsulinemia. The db/db mouse, a more severely hyperglycemic animal, develops progressive insulin depletion in the pancreas.

The thiazolidinediones are a new class of antihyperglycemic agents that appear to enhance insulin sensitivity without stimulating pancreatic insulin release.[5] Thiazolidinediones are known to improve the impairment of the insulin-stimulated glucose disposal rate in vivo in insulin-resistant animals.[6,7] However, the effect of thiazolidinediones on basal glucose uptake in skeletal muscle from insulin-resistant animals remains poorly understood.

Recently, we identified a new thiazolidinedione, KRP-297, (±)-5-[(2,4-dioxothiazolidin-5yl)methyl]-2-methoxy-*N*-[[4-(trifluoromethyl)phenyl]methyl] benzamide, which acts as a coligand for the α and γ isoforms of peroxisome proliferator–activated receptors (PPARs),[8] unlike the classic thiazolidinediones, which are PPARγ-selective ligands.[9,10] In the present study, we characterized the effect of KRP-297 on basal and insulin-stimulated glucose uptake using soleus muscle isolated from ob/ob mice and db/db mice. Moreover, to examine the effect of KRP-297 on the development of diabetes in db/db mice, pancreatic insulin content was measured.

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MATERIALS AND METHODS

Chemicals

KRP-297 was synthesized by Kyorin Pharmaceutical Co Ltd (Tochigi, Japan). [1-^{14}C]2-deoxyglucose (2DG) and [^{3}H]L-glucose were obtained from New England Nuclear (Boston, MA). Porcine insulin and bovine serum albumin ([BSA] fraction V) were purchased from Sigma Chemical (St Louis, MO).

Animals

Male C57BL/6J-ob/ob mice, C57BL/Ks-db/db mice, and lean control mice were obtained from Charles River Japan (Yokohama, Japan). All mice received standard chow (Japan Clea, Tokyo, Japan) and tap water ad libitum. All institutional guidelines for animal care and use were followed in this study.

At the start of KRP-297 or control solution administration, ob/ob mice (n = 6) and lean control mice (n = 6) were 9 weeks of age. Db/db mice (n = 6) and their lean controls (n = 6) were used at 8 weeks of age. KRP-297 or a control vehicle (0.5% gum arabic solution) were administered orally to the mice once per day. Mice that were fasted for 20 hours were subjected to an oral glucose tolerance test after receiving glucose solution (2 g/kg), and blood samples were collected at 0, 30, 60, and 120 minutes after the glucose load. A plasma sample was centrifuged, and the supernatant was assayed for glucose and insulin. Glucose and insulin levels were determined by a glucose B-test and an insulin EIA-test (Wako Pure Chemical Industries, Osaka, Japan), respectively.

Measurement of 2DG Uptake

At the end of the treatment period, the soleus muscles were isolated. 2DG uptake was studied using the methods of Le Marchand-Brustel et al,[11] which we partially modified as follows. The soleus muscles were removed from the hindlimbs, ligated around each tendon using silk surgical thread, and attached across a plastic holder. The muscles were washed for 15 minutes at 25°C in Krebs-Ringer phosphate buffer (pH 7.4) containing 10 mmol/L HEPES, 1% BSA, and 2 mmol/L sodium pyruvate (buffer A). They were washed three times in buffer A, preincubated for 120 minutes at 25°C in buffer A with or without insulin (2.5 mU/mL), transferred to fresh buffer A containing 2-deoxy-D-[1-^{14}C]glucose ([2DG] 0.15 μCi/muscle) and L-[1-^{3}H]glucose (1.5 μCi/muscle) with or without insulin (2.5 mU/ml), and incubated for 30 minutes at 25°C. At the end of the preincubation, the muscles were washed three times in chilled buffer A, weighed, and then dissolved in 2N NaOH. They were neutralized with 2N HCl and dissolved in ACS II (Amersham, Buckinghamshire, UK). ^{14}C and ^{3}H specific activities were counted in a liquid scintillation counter (Packard Instrument, Meriden,

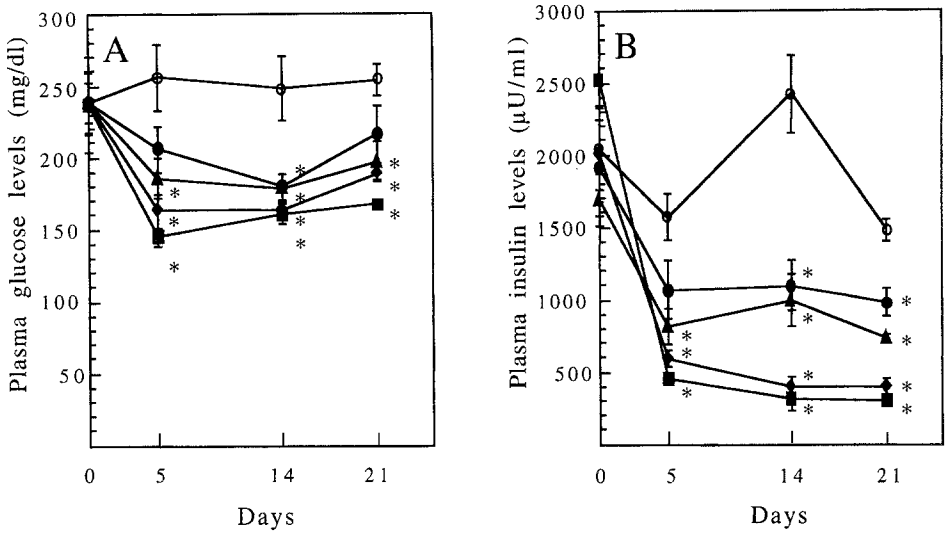

Fig 1. Changes in plasma glucose and insulin levels in KRP-297–treated and untreated ob/ob mice. Values are the mean ± SE (n = 6). *$P < .05$ *v* ob/ob control mice, ○, control; ●, 0.3 mg/kg; ▲, 1 mg/kg; ◆, 3 mg/kg; ■, 10 mg/kg.

CT). All incubations were performed after gassing with O_2:CO_2 (95%:5%) in a shaking incubator. The specific uptake of 2DG was calculated by subtracting the nonspecific uptake of L-glucose from the total uptake of 2DG.

Determination of Pancreatic Insulin

Frozen aliquots of the pancreas were stored at −80°C until all specimens were available. Each pancreatic specimen was homogenized in 1.8 mL acid ethanol (0.15 mol/L HCl, 75% EtOH). The homogenates were stored at 4°C for 48 hours and then centrifuged at 9,000 × *g* for 10 minutes at 4°C. The supernatants were pooled, and the pellets were then resuspended in 0.2 mL acid ethanol. After centrifugation, the first and second supernatants were pooled and then diluted 1:2,000 in 0.1 mol/L phosphate-saline buffer containing 0.25% BSA. The insulin level was determined using an insulin EIA-test (Wako Pure Chemical Industries).

Statistical Analysis

Results obtained from these animal studies are presented as the mean ± SE. The unpaired Student's *t* test and Dunnett's test were used for statistical evaluation.

RESULTS

Effects of KRP-297 in ob/ob Mice

KRP-297 (0.3 to 10 mg/kg) was administered orally to ob/ob mice for 21 days. Under the same conditions, to investigate the specificity of action, lean mice were also treated with 10 and 100 mg/kg KRP-297 for 21 days. KRP-297 (0.3 to 10 mg/kg) decreased plasma glucose and insulin in a dose-dependent manner (Fig 1). The glucose and insulin–lowering effect of KRP-297 reached a plateau after 5 days of treatment. An excess dose of KRP-297 showed no effect on plasma glucose levels in lean mice (Fig 2). At 15 days after KRP-297 treatment, fasting ob/ob mice were subjected to an oral glucose tolerance test. KRP-297 decreased plasma glucose and insulin levels before (during fasting) and after the glucose load in a dose-dependent manner (Fig 3).

To evaluate the effect of KRP-297 on the defect in the peripheral glucose disposal rate in ob/ob mice, soleus muscles were isolated from KRP-297–treated or untreated ob/ob mice and untreated lean mice and basal and insulin-stimulated 2DG uptake was measured (Table 1). Basal 2DG uptake was slightly (10.6%) lower in ob/ob mice versus lean mice, although the difference was not statistically significant. Insulin produced a 133% increase in 2DG uptake in lean mice but only a slight increase (20%) in ob/ob mice. Insulin-stimulated 2DG uptake was 53.8% lower ($P < .05$) in ob/ob mice than in lean mice. KRP-297 had no significant effect on basal 2DG uptake but it restored the reduced insulin-stimulated 2DG uptake in soleus muscle from ob/ob mice in a dose-dependent manner.

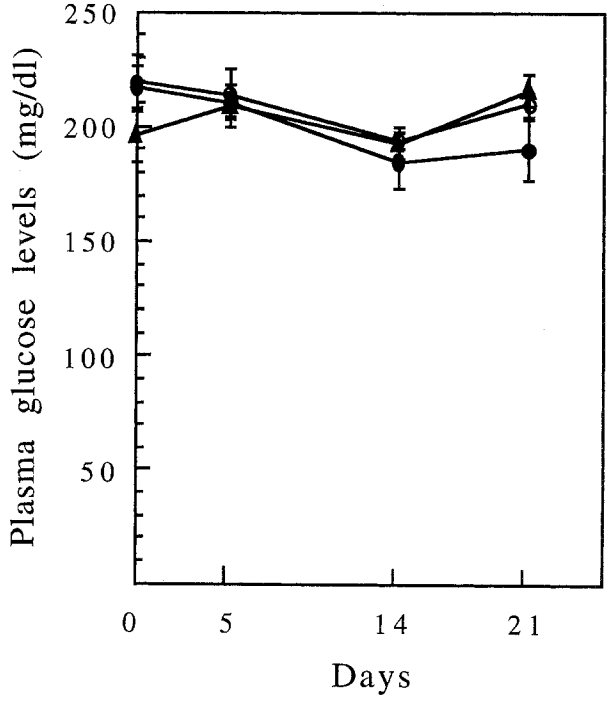

Fig 2. Effect of KRP-297 on plasma glucose levels in lean mice. Values are the mean ± SE (n = 6). ○, control; ●, 10 mg/kg; ▲, 100 mg/kg.

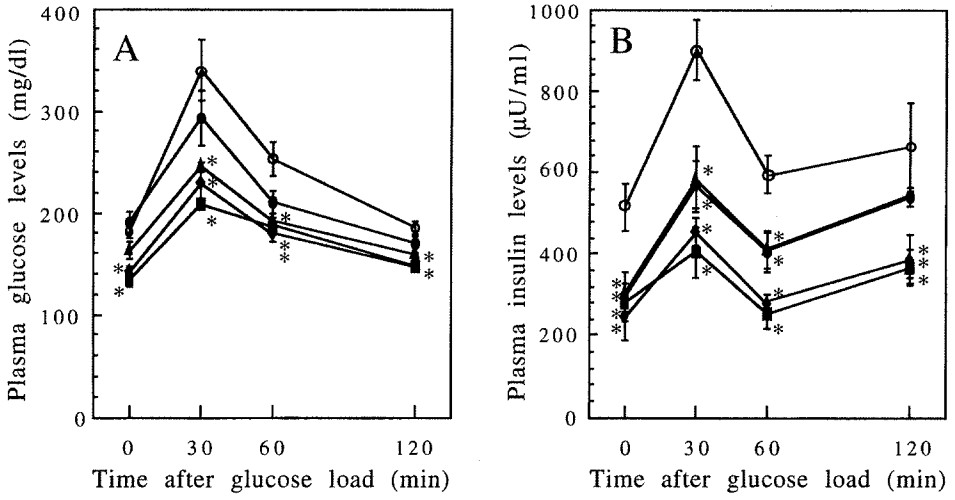

Fig 3. Changes in plasma glucose and insulin levels during oral glucose tolerance test. At 15 days after KRP-297 treatment, fasting mice were subjected to an oral glucose (1 g/kg) tolerance test and plasma glucose (A) and insulin (B) were measured. Values are the mean ± SE (n = 6). *$P < .05$ *v* ob/ob control mice. ○, control; ●, 0.3 mg/kg; ▲, 1 mg/kg; ◆, 3 mg/kg; ■, 10 mg/kg.

Study in db/db Mice

Plasma glucose levels in db/db mice were decreased at 28 days after KRP-297 treatment (0.3 to 10 mg/kg) in a dose-dependent manner (Fig 4A). At 7 and 14 days after KRP-297 treatment, plasma insulin levels were also decreased in a dose-dependent manner, whereas there was no difference in plasma insulin between KRP-297–treated and vehicle-treated db/db mice at 28 days (Fig 4B). At 28 days after KRP-297 treatment, pancreatic insulin content was markedly lower in db/db mice than in lean mice ($P < .01$; Fig 5), suggesting that db/db mice exhibit an increase in compensatory insulin release for hyperglycemia. KRP-297 restored the pancreatic insulin content in db/db mice in a dose-dependent manner.

Table 2 shows 2DG uptake in soleus muscle from db/db mice. The insulin-stimulated increases were 84% in lean mice and 40% in ob/ob mice, respectively. Basal 2DG uptake showed a profound decrease (35.0%, $P < .01$) in db/db mice compared with lean mice. Insulin-stimulated 2DG uptake was 50.5% lower in db/db mice versus lean mice ($P < .01$). When administered to db/db mice for 28 days, KRP-297 restored basal and insulin-stimulated 2DG uptake in a dose-dependent manner.

DISCUSSION

We examined the effect of KRP-297, a dual agonist for PPARα and PPARγ, in insulin-resistant animals with moderate or severe hyperglycemia. In ob/ob mice, KRP-297 improved moderate hyperglycemia and severe hyperinsulinemia. The antihyperglycemic effect of KRP-297, unlike that of the sulfonylureas, appears to be confined to these insulin-resistant mice, and no such effect is found in normal mice. In this study, soleus muscle from ob/ob mice exhibited a lower insulin-stimulated 2DG uptake, suggesting the presence of peripheral insulin resistance. This defect was improved by treatment with KRP-297. To date, the mechanism of impaired basal 2DG uptake in soleus muscle from ob/ob mice remains controversial. Cuendet et al[2] have reported a significant decrease in the soleus muscles, while other groups have shown only a slight decrease,[11,12] in agreement with our data. Studies using perfused hindquarters have shown that treatment with other thiazolidinediones produces an increase or no effect in basal glucose disposal rates in various animal models of insulin resistance with moderate hyperglycemia such as obese Zucker fatty rats,[13] high-fructose–fed rats,[14] and ob/ob mice.[15]

We have shown here that basal glucose uptake is reduced more in db/db mice than in ob/ob mice, consistent with a previous report using the perfused hindquarters.[3] An impairment in basal glucose uptake was also reported in insulin-deficient animals such as the streptozotocin-induced diabetic rat, which has severe hyperglycemia.[16] Glucosamine reduced basal and insulin-stimulated glucose uptake in soleus muscle in vitro,[17] suggesting the involvement of a glucosamine biosynthetic pathway induced by hyperglycemia. Our results support the hypothesis that an elevation of the circulating glucose level may be important for the reduction of basal glucose uptake in skeletal muscle. Furthermore, this study shows that KRP-297 ameliorated the severe hyperglycemia in db/db mice, and this effect was associated with an improvement of the impaired basal and insulin-stimulated 2DG uptake in soleus muscle. At 28 days after KRP-297 treatment, pancreatic insulin content was lower in db/db mice than in lean mice. Plasma insulin levels in db/db mice were lower at 28 days versus 7 and 14 days, and plasma glucose levels progressively increased with age. The reduced pancreatic insulin content in db/db mice may indicate that insulin synthesis is inadequate to meet the demand for insulin secretion in adaptation to prolonged hyperglycemia. Treatment with KRP-297 produced a marked improvement in

Table 1. Effect of KRP-297 on 2DG Uptake in Soleus Muscle From ob/ob Mice

Mice	Treatment	Dose (mg/kg)	2DG Uptake (nmol/g/30 min)	
			Basal	Insulin-Stimulated
Lean	Vehicle	—	124.1 ± 2.6	288.9 ± 12.7*
Ob/ob	Vehicle	—	110.9 ± 5.6	133.6 ± 3.8
Ob/ob	KRP-297	0.3	111.3 ± 6.5	142.5 ± 7.8
Ob/ob	KRP-297	1	115.3 ± 9.6	183.0 ± 10.5†
Ob/ob	KRP-297	3	121.4 ± 6.5	199.3 ± 9.1†
Ob/ob	KRP-297	10	127.5 ± 6.7	213.3 ± 16.8†

NOTE. The insulin concentration is 2.5 mU/mL. Data are the mean ± SE of 5 or 6 mice.

*$P < .05$, †$P < .01$ *v* ob/ob control (vehicle).

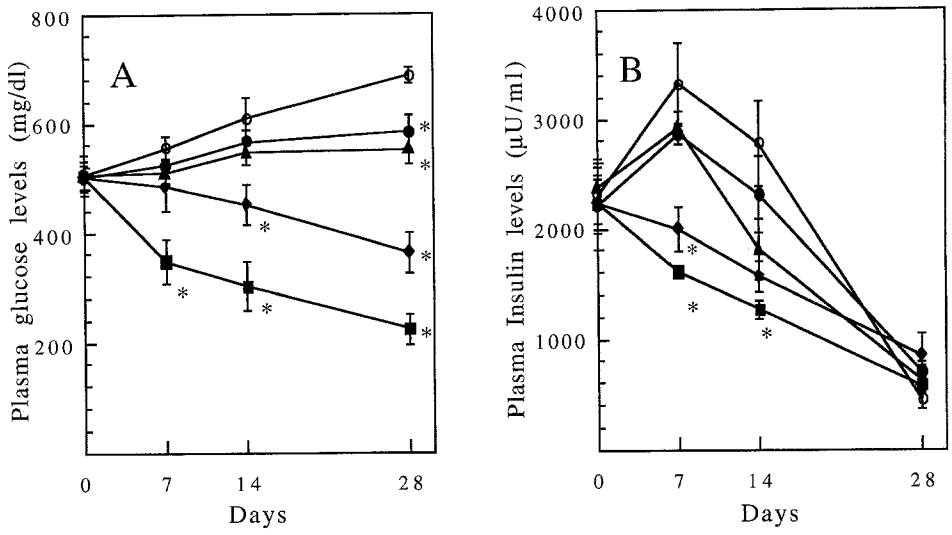

Fig 4. Changes in plasma glucose and insulin levels in KRP-297–treated and untreated db/db mice. Values are the mean ± SE (n = 6). *$P < .05$ *v* db/db control mice. ○, control; ●, 0.3 mg/kg; ▲, 1 mg/kg; ◆, 3 mg/kg; ■, 10 mg/kg.

the pancreatic insulin depletion in db/db mice. KRP-297 improved the hyperglycemia and hyperinsulinemia in db/db mice at 7 and 14 days, suggesting that KRP-297 decreased the demand for insulin secretion in the early diabetic stage. Accordingly, the effect of KRP-297 on insulin content might be explained, at least in part, by an amelioration of the hyperglycemia, as previously demonstrated in other thiazolidinedione-treated or diet-restricted db/db mice.[18-20]

The mechanisms by which KRP-297 improves the impaired glucose uptake in skeletal muscle and the pancreatic insulin depletion in db/db mice are unknown. Skeletal muscle has been shown to express PPARα and PPARγ1 in addition to PPARδ.[21] In the pancreas, PPARα was recently suggested to function as a regulator of lipid oxidation.[22] Therefore, we cannot exclude the possibility that KRP-297 acts directly on skeletal muscle and the pancreas. Elevated lipid levels have been implicated in the development of peripheral insulin resistance and β-cell dysfunction.[23] We previously reported that treatment with KRP-297 reduces cellular triglyceride levels in the liver, a tissue with abundant PPARα, in obese Zucker fatty rats.[8] Further studies are needed to investigate the effect of KRP-297 on lipid metabolism in the skeletal muscle and pancreas of db/db mice.

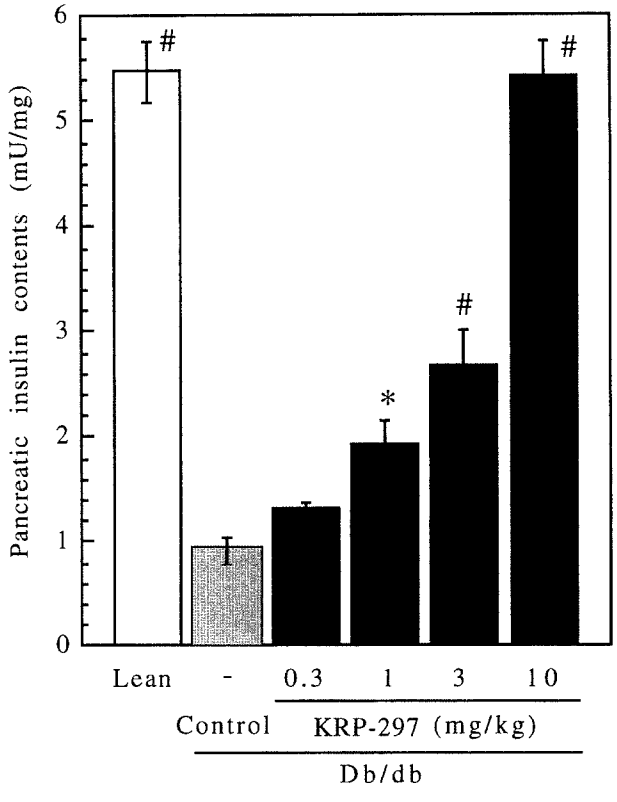

Fig 5. Effect of KRP-297 on pancreatic insulin content in db/db mice. Values are the mean ± SE (n = 6). *$P < .05$, #$P < .01$ *v* db/db control mice.

In summary, KRP-297, a dual agonist of PPARα and PPARγ, had antihyperglycemic effects and restored both basal and insulin-stimulated glucose uptake in skeletal muscle in insulin-resistant mice with severe hyperglycemia. Moreover, KRP-297 prevented the marked pancreatic insulin depletion in these mice. These results suggest that KRP-297 treatment is useful to prevent the development of diabetic syndromes in the insulin-resistant NIDDM associated with obesity. To better understand the effect of the dual agonism of PPARα and PPARγ, the effects of KRP-297 treatment should be compared with PPARγ-selective ligands, ie, other thiazolidinediones.

Table 2. Effect of KRP-297 on 2DG Uptake in Soleus Muscle From db/db Mice

Mice	Treatment	Dose (mg/kg)	2DG Uptake (nmol/g/30 min)	
			Basal	Insulin-Stimulated
Lean	Vehicle	—	55.7 ± 0.1*	102.7 ± 0.3*
Db/db	Vehicle	—	36.2 ± 0.1	50.8 ± 0.1
Db/db	KRP-297	0.3	45.4 ± 0.2*	70.5 ± 0.2*
Db/db	KRP-297	1	50.8 ± 3.8*	89.0 ± 11.7*
Db/db	KRP-297	3	52.3 ± 0.2*	95.2 ± 0.4*
Db/db	KRP-297	10	54.2 ± 0.3*	102.9 ± 0.6*

NOTE. The insulin concentration is 2.5 mU/mL. Data are the mean ± SE of 5 or 6 mice.

*$P < .01$ *v* db/db control (vehicle).

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