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European Journal of Pharmacology 304 (1996) 129–134

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Amelioration of insulin resistance in genetically obese rodents by M16209, a new antidiabetic agent

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Received 17 January 1996; revised 12 February 1996; accepted 13 February 1996

Abstract

Improvement of metabolic disorders by M16209 (1-(3-bromobenzofuran-2-ylsulfonyl)hydantoin), an antidiabetic agent, was studied in genetically obese Zucker fa/fa rats and C57BL/6J ob/ob mice. In fa/fa rats oral administration of M16209 (30 and 100 mg/kg/day) for 7 days dose dependently improved hyperinsulinemia without affecting body weight. Oral glucose loading (2 g glucose/kg body weight) after 10 days of administration to fa/fa rats revealed that M16209 significantly improved glucose tolerance both 30 and 60 min after glucose loading, but did not affect preload serum glucose levels. At one day after 13 days of administration of M16209, the serum levels of triglyceride, total cholesterol and free fatty acid were clearly lower in treated fa/fa rats than those in untreated rats. In C57BL/6J ob/ob mice, M16209 given for 28 days at doses of 30 and 100 mg/kg/day improved hyperinsulinemia, hyperglycemia and hypercholesterolemia without affecting body weight. In a hyperinsulinemic euglycemic clamp study in fa/fa rats, administration of M16209 for 7 days at a dose of 100 mg/kg/day significantly normalized the decreased metabolic clearance rate but did not show any effect on the augmented hepatic glucose output. These findings demonstrate that improvement of metabolic disorders in genetically obese rodents by M16209 is due to amelioration of insulin resistance in peripheral tissues.

Keywords: M16209; Insulin resistance; Genetically obese rodent; Hyperinsulinemic euglycemic clamp; Antihyperglycemic effect

1. Introduction

We previously demonstrated that a single oral administration of M16209 (1-(3-bromobenzofuran-2-ylsulfonyl)hydantoin) (see Fig. 1 for chemical structure), a potent aldose reductase inhibitor (Kato et al., 1990, 1991a,b), had antihyperglycemic effects in rats with streptozotocin-induced mild diabetes and neonatally streptozotocin-induced, non-insulin-dependent diabetes mellitus (Nakayama et al., 1995a). M16209 has been reported to augment glucose-stimulated insulin secretion in rats via a direct effect on the pancreas (Nakayama et al., 1995b). This is thought to be one of the mechanisms of the antihyperglycemic acute effect of M16209. However, involvement of an extrapancreatic action of M16209 in antihyperglycemic activity remains unclear.

In this study, we therefore investigated the effect of chronic treatment with M16209 on Zucker fa/fa rats and C57BL/6J ob/ob mice, which are genetically obese, and in diabetic and hyperinsulinemic rodents with insulin resistance. In addition, we performed a hyperinsulinemic euglycemic clamp study in Zucker fa/fa rats treated chronically with M16209 in order to elucidate the mechanism of the extrapancreatic effect of M16209.

2. Materials and methods

2.1. Antidiabetic study in Zucker fa/fa rats

Female Zucker fa/fa rats and their lean littermates (fa/?) were purchased from Charles River Kingston

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PII S0014-2999(96)00121-5

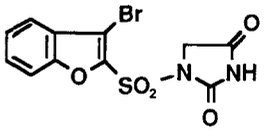

Fig. 1. Chemical structure of M16209.

(Kingston, NY, USA) and used from 16 weeks of age. Zucker fa/fa rats were divided into three groups of five each in such a fashion that body weight and serum glucose level did not differ significantly among the groups. Five lean littermates were also used. The rats were maintained on laboratory chow and water ad libitum, and housed in a temperature- and humidity-controlled (23 ± 2°C, 55 ± 5%) room lighted from 7:00 to 20:00 h. Body weight was measured daily. M16209 was suspended in 5% gum arabic solution and administered orally once daily for 13 days at doses of 30 or 100 mg/kg/day, and control rats were given vehicle alone. The lean littermates were given no treatment. Six hours after administration on the 7th day, blood samples were taken from the retro-orbital sinus and sera were separated for determination of insulin levels. Oral glucose tolerance was tested on the 10th day of administration in 17-h-fasted Zucker fa/fa rats and their lean littermates. Three hours after the administration of M16209, glucose (2 g/kg) was loaded orally. Blood samples were taken from the retro-orbital sinus just before and at 30, 60, 120 and 240 min after glucose loading. Sera were separated for determination of glucose and insulin. One day after the last administration, rats were killed by decapitation, blood samples were obtained and sera were separated for determination of triglyceride, total cholesterol and free fatty acid. The total cholesterol assay was done with chloroform-methanol (2:1) extracts of sera. Serum glucose and total cholesterol were assayed using colorimetric kits based on the glucose oxidase and cholesterol oxidase methods, respectively. Serum triglyceride and free fatty acid were assayed using colorimetric kits based on the acetylacetone and modified Duncombe methods, respectively. These kits were obtained from Wako Pure Chemical Industries (Osaka, Japan). Serum insulin was assayed using an enzyme immunoassay kit from Sanko Junyaku Co. (Tokyo, Japan).

2.2. Antidiabetic study in C57BL/6J ob/ob mice

Female C57BL/6J ob/ob mice and their lean littermates (ob/+) were purchased from Jackson Laboratory (Bar Harbor, MA, USA) and were used at 8 weeks of age and maintained as described above. C57BL/6J ob/ob mice were divided into three groups of nine or ten each in such a fashion that body weight and serum glucose level did not differ significantly among the groups. Seven lean littermates were also used. M16209 was administered orally once daily for 28 days at doses of 30 or 100 mg/kg/day to C57BL/6J ob/ob mice, and vehicle alone was administered to the controls. Nothing was administered to lean littermates. Body weight was measured daily and food intake was measured between day 18 and 20. Just before the first and the 14th administrations and one day after the last administration, blood samples were taken from the retro-orbital sinus, and sera were separated for determination of glucose, insulin, triglyceride, total cholesterol and free fatty acid. Serum triglyceride, total cholesterol and free fatty acid were assayed using colorimetric kits based on the glycerol-3-phosphate oxidase method, cholesterol oxidase method and acyl-CoA oxidase method, respectively. The kits were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Hyperinsulinemic euglycemic clamp study

Male Zucker fa/fa and their lean littermates were used at 8 weeks of age and maintained as described above. Zucker fa/fa rats were divided into two groups of six and eight in such a fashion that neither body weight nor serum glucose differed significantly between the groups. Six lean littermates were used. M16209 was administered orally once daily for 7 days at a dose of 100 mg/kg/day to Zucker fa/fa rats, and vehicle alone was administered to the controls. Nothing was administered to the lean littermates. Food intake was measured between day 5 and 7. One day after the last administration, 17-h-fasted rats were anesthetized with the intraperitoneal administration of 52 mg/kg α-chloralose and 640 mg/kg urethane. The left carotid artery (for blood sampling), right jugular vein (for 3-[^{3}H]glucose infusion), left femoral vein (for exogenous glucose infusion) and right femoral vein (for insulin infusion) were catheterized with fine silicon tubing (Silastic, Dow-Corning Corp., Midland, MI, USA) filled with 50 U/ml heparin. A tracheotomy was routinely done to avoid respiratory problems during anesthesia. The rats were kept on a heating stand (37°C) to maintain body temperature throughout the study. The hyperinsulinemic euglycemic clamp study with 3-[^{3}H]glucose infusion was performed using the method of Kergort and Portha (1985) with some modifications in an attempt to determine whole-body insulin sensitivity and hepatic glucose output. A priming (48.1 kBq) and a continuous (1.1 kBq/min) infusion of 3-[^{3}H]glucose (DuPont/NEN, Boston, MA, USA) were initiated and continued throughout the experiment. Forty to 45 min after the start of labeled glucose infusion, 30 mU/kg/min insulin (Actrapid, NOVO, Copenhagen, Denmark) was infused in the priming mode (initially 60 mU/kg/min for 10 min) to suspend hyperinsulinemia for 75 min. Blood samples (30 μl) were taken every 5 min, and plasma glucose concentrations were measured with ANTSENSE (Bayer Sankyo, Tokyo, Japan), a table-top blood glucose analyzer based on the immobilized enzyme membrane/H_2O_2 electrode method. The specific activity of labeled glucose was measured, and the glucose disposal

rate, metabolic clearance rate of glucose and hepatic glucose output were calculated using the methods of Steele (1959) and Radziuk and Lickley (1985).

2.4. Statistical analysis

Values are expressed as means ± S.E. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Bonferroni method or Student's *t*-test for data with equal variances. When the assumption of homogeneity of variances was violated (Bartlett's test), the data were analyzed using the Kruskal-Wallis test followed by the Dunnett type mean-rank test, Aspin-Welch *t*-test or Cochran-Cox *t*-test.

3. Results

3.1. Studies on Zucker fa / fa rats

Zucker fa/fa rats were remarkably obese compared with lean littermates. M16209 at doses of 30 and 100 mg/kg/day had no effect on body weight. The final mean body weight (g) in each group was as follows: control 556 ± 21; 30 mg/kg/day M16209, 556 ± 21; 100 mg/kg/day M16209, 546 ± 18; lean littermates 262 ± 10.

As shown in Fig. 2, serum insulin levels in control fa/fa rats were far higher than those in lean littermates. M16209 at doses of 30 and 100 mg/kg/day decreased serum insulin level in a dose-dependent fashion in fa/fa rats.

Fig. 3A shows the glucose tolerance curves. The serum glucose levels in control fa/fa rats were significantly higher than those in lean littermates up to 4 h after glucose loading. The serum glucose levels of fa/fa rats given M16209 at doses of 30 and 100 mg/kg/day were low compared with those of vehicle-treated fa/fa rats at both 0.5 and 1 h after glucose loading. The preloading glucose levels in control fa/fa rats were slightly but significantly higher than those in lean littermates, whereas there was no difference in preloading glucose level between vehicle- and M16209-treated fa/fa rats. The serum insulin levels in

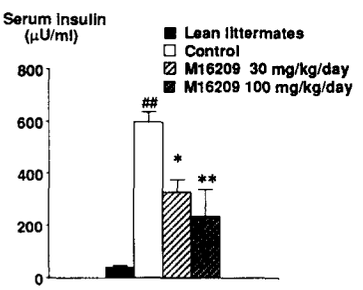

Fig. 2. Effect of 7-day administration of M16209 on serum insulin level in genetically obese Zucker fa/fa rats. Blood samples were collected 6 h after the final administration of M16209. Values are means ± S.E. ($n = 5$). $^{\#\#}$ $P < 0.01$ vs. lean littermates, ** $P < 0.01$, * $P < 0.05$ vs. control.

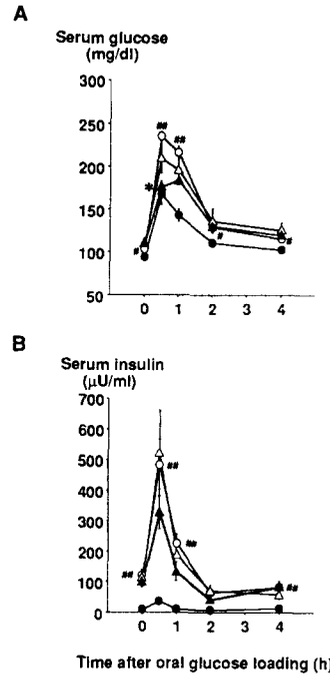

Fig. 3. Effects of 10-day administration of M16209 on serum glucose level (A) and serum insulin level (B) in genetically obese Zucker fa/fa rats given orally 2 g/kg glucose. Glucose was given 3 h before the final administration of M16209. Control (○), 30 mg/kg/day M16209 (△), 100 mg/kg/day M16209 (▲), lean littermates (●). Values are means ± S.E. ($n = 5$). $^{\#\#}$ $P < 0.01$, $^{\#}$ $P < 0.05$ vs. lean littermates, * $P < 0.05$ vs. control.

control fa/fa rats were significantly higher than those in lean littermates both before and after glucose loading (Fig. 3B). The serum insulin levels in fa/fa rats treated with M16209, 100 mg/kg/day, but not 30 mg/kg/day, were lower than those in the control group at 0.5 and 1 h after glucose loading.

Table 1 summarizes the serum lipid levels in Zucker fa/fa rats treated orally with M16209 or vehicle and in lean littermates. The serum triglyceride, total cholesterol and free fatty acid levels in control fa/fa rats were 23, 3.5 and 2.1 times the respective level in lean littermates. M16209 at 100 mg/kg/day, but not 30 mg/kg/day, lowered these serum lipid levels.

3.2. Studies on C57BL / 6J ob / ob mice

C57BL/6J ob/ob mice were obese compared with lean littermates. The final mean body weight (g) in each group was as follows: control, 50.9 ± 1.1; 30 mg/kg/day M16209, 51.0 ± 2.4; 100 mg/kg/day M16209, 50.3 ± 2.0; lean littermates, 20.8 ± 0.3. M16209 at doses of 30 and 100 mg/kg/day had no effect on body weight. The food intake (g/mouse/day) between day 18 and 20 in each group was as follows: control, 5.7 ± 0.2; 30 mg/kg/day M16209, 5.8 ± 0.2; 100 mg/kg/day M16209, 5.4 ± 0.2; lean littermates, 3.3 ± 0.1. M16209 at

doses of 30 and 100 mg/kg/day had no effect on food intake.

Fig. 4A,B show the serum levels of glucose and insulin, respectively, in fed C57BL/6J ob/ob mice and their lean littermates on the 14th day of administration. M16209 decreased serum glucose and insulin levels in a dose-dependent fashion. Moreover, these antihyperglycemic and antihyperinsulinemic effects of M16209 were also observed on the 29th day of administration (data not shown).

Table 2 summarizes the serum lipid levels in ob/ob mice treated orally with M16209 or vehicle and in lean littermates. Serum levels of triglyceride and total cholesterol in control ob/ob mice were higher than those in lean littermates, while the serum free fatty acid level was almost the same in the two groups. M16209 at a dose of 100 mg/kg/day significantly decreased the total cholesterol level, but the drug had no effect on the triglyceride and free fatty acid levels.

3.3. Hyperinsulinemic euglycemic clamp study

Table 3 shows the plasma glucose and insulin levels at basal and steady state in fa/fa rats pretreated orally with M16209 or vehicle for 7 days and in lean littermates. The final mean body weight (g) in each group was as follows: control, 391 ± 7 (g); 100 mg/kg/day M16209, 396 ± 11; lean littermates, 293 ± 10. The food intake (g/rat/day) between day 5 and 7 in each group was as follows: control, 36.5 ± 1.0; 100 mg/kg/day M16209, 38.0 ± 2.6; lean littermates, 24.7 ± 0.7. M16209 at a dose of 100 mg/kg/day had no effect on food intake. In the basal state, the plasma levels of glucose and insulin in control fa/fa rats were significantly higher than those in lean littermates, and were decreased by treatment with M16209. At steady state, there was no significant difference in either glucose or insulin levels among control, M16209-treated and lean groups.

Table 1
Effects of 13-day administration of M16209 on serum lipid levels in genetically obese Zucker fa/fa rats

Group	TG (mg/dl)	T-Chol (mg/dl)	FFA (μEq/l)
Lean littermates	148 ± 35	68 ± 1	492 ± 88
fa/fa			
Control	3430 ± 750 [b]	235 ± 43 [b]	1030 ± 62 [a]
M16209 (30 mg/kg/day)	3110 ± 1320	248 ± 75	802 ± 63
M16209 (100 mg/kg/day)	480 ± 79 [c]	149 ± 4 [d]	576 ± 80 [c]

Values are means ± S.E. [a] $P < 0.01$, [b] $P < 0.05$ vs. lean littermates, [c] $P < 0.01$, [d] $P < 0.05$ vs. control. Abbreviations: TG, triglyceride; T-Chol, total cholesterol; FFA, free fatty acid.

Table 2
Effects of 28-day administration of M16209 on serum lipid levels in genetically obese C57BL/6J ob/ob mice

Group	*n*	TG (mg/dl)	T-Chol (mg/dl)	FFA (μEq/l)
Lean littermates	7	128 ± 9	98 ± 4	1795 ± 120
ob/ob				
Control	10	201 ± 19 [a]	286 ± 9 [a]	2074 ± 87
M16209 (30 mg/kg/day)	10	180 ± 11	260 ± 7	1910 ± 50
M16209 (100 mg/kg/day)	9	244 ± 19	258 ± 8 [b]	1969 ± 100

Values are means ± S.E. [a] $P < 0.01$ vs. lean littermates, [b] $P < 0.05$ vs. control. Abbreviations: TG,triglyceride; T-Chol, total cholesterol; FFA, free fatty acid.

Table 3
Effects of 7-day administration of M16209 on plasma insulin and glucose levels during hyperinsulinemic euglycemic clamp study in Zucker fa/fa rats

	Group	*n*	Glucose (mg/dl)	Insulin (μU/ml)
Basal	Lean littermates	6	118 ± 5	33 ± 4
	fa/fa			
	Control	8	208 ± 15 [a]	144 ± 10 [a]
	M16209 (100 mg/kg/day)	6	123 ± 6 [b]	85 ± 5 [b]
Insulin (30 mU/kg/min)	Lean littermates	6	124 ± 4	2676 ± 174
	fa/fa			
	Control	4	113 ± 3	3414 ± 374
	M16209 (100 mg/kg/day)	6	120 ± 3	3113 ± 252

Values are means ± S.E. [a] $P < 0.01$ vs. lean littermates, [b] $P < 0.01$ vs. control.

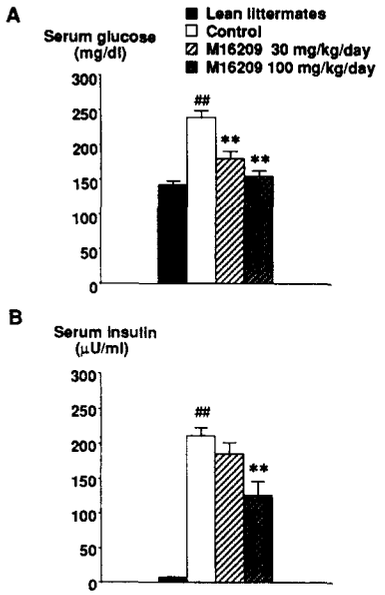

Fig. 4. Effects of 13-day administration of M16209 on serum glucose level (A) and serum insulin level (B) in genetically obese C57BL/6J ob/ob mice. Blood samples were collected one day after the final administration of M16209. Values are means ± S.E. ($n = 7–10$). $^{\#\#} P <$ 0.01 vs. lean littermates, $^{**} P < 0.01$ vs. control.

Fig. 5A,B,C show glucose disposal rate, metabolic clearance rate of glucose and hepatic glucose output, respectively, in the hyperinsulinemic euglycemic clamp study in fa/fa rats and in their lean littermates. In the basal state, glucose disposal rate and hepatic glucose output were significantly higher in control fa/fa rats than in lean littermates, but the metabolic clearance rate of glucose was the same in the two groups. The metabolic clearance rate of glucose but neither glucose disposal rate nor hepatic glucose output was increased by administration of M16209. In the hyperinsulinemic state, glucose disposal rate and metabolic clearance rate of glucose were lower and hepatic glucose output was higher in control fa/fa rats than in lean littermates. M16209 increased glucose disposal rate and metabolic clearance rate of glucose to the levels observed in lean littermates, but had no effect on hepatic glucose output.

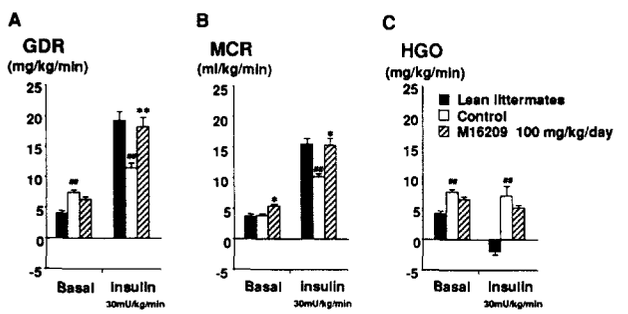

Fig. 5. Effects of 7-day administration of M16209 on GDR (A), MCR (B) and HGO (C) during a hyperinsulinemic (30 mU/kg/min) euglycemic clamp study in Zucker fa/fa rats. This study was performed one day after the final administration of M16209. Values are means ± S.E. ($n = 4–8$). $^{\#\#} P < 0.01$ vs. lean littermates, $^{**} P < 0.01$, $^{*} P < 0.05$ vs. control. Abbreviations: GDR, glucose disposal rate; MCR, metabolic clearance rate of glucose; HGO, hepatic glucose output.

4. Discussion

Non insulin-dependent diabetes mellitus (NIDDM) is usually accompanied by insulin resistance. Zucker fa/fa rats and C57BL/6J ob/ob mice are genetically obese and insulin resistant rodents with hyperinsulinemia. The former also manifest glucose intolerance and severe hyperlipidemia, while the latter exhibit hyperglycemia and mild hyperlipidemia (Shafrir, 1992). In this study, we found that chronic treatment with M16209 dose dependently improved glucose intolerance in fa/fa rats and hyperglycemia in ob/ob mice.

We had previously demonstrated that enhancement of glucose-stimulated insulin secretion is one of the mechanisms of the antihyperglycemic effect of M16209. This effect of M16209 was observed even at 6 h after administration in an in vivo study and also just after glucose stimulation in isolated, perfused rat pancreas (Nakayama et al., 1995a,b). On the contrary, in the present study, chronic administration of M16209 to fa/fa rats decreased the serum insulin level. Also, chronic treatment with M16209 lowered both serum insulin and glucose levels in ob/ob mice. These findings strongly suggest that, in genetically insulin-resistant rodents, the antihyperglycemic effect of M16209 is not due to the enhancement of glucose-stimulated insulin secretion and is caused instead by amelioration of insulin resistance. Therefore the acute antihyperglycemic effect of M16209 is mainly caused by the insulinotropic action, while the peripheral action of M16209 may be a result of chronic treatment.

Hyperlipidemia, in particular hypertriglyceridemia, is one of the most prominent features of Zucker fa/fa rats. This study showed that the abnormality was markedly improved by chronic treatment with M16209. The association of hyperlipidemia with insulin resistance has been documented: the increase in hepatic very-low-density lipoprotein (VLDL)-triglyceride secretion caused by hyperinsulinemia and the reduction of the rate of removal of VLDL-triglyceride from plasma that accompanies insulin resistance has been shown to cause hypertriglyceridemia (Reaven and Greenfield, 1981; Modan et al., 1988). It is thus conceivable that the amelioration of hyperlipidemia by M16209 is due to the improvement of hyperinsulinemia through augmentation of insulin sensitivity. Moreover, it is likely that the reduction of plasma free fatty acid levels by M16209 might contribute to the improvement of insulin resistance in fa/fa rats, since high free fatty acid has been reported to cause insulin resistance and glucose intolerance by inhibiting glycolysis and glucose transport in muscle (Pelkonen et al., 1968; Thiebaud et al., 1982). The large drop of serum triglyceride levels in fa/fa rats by M16209

may be due to the reduction of accelerated VLDL secretion through amelioration of the hyperinsulinemia as well as the amelioration of impaired VLDL breakdown through increased peripheral insulin sensitivity. Since cholesterol is less abundant in VLDL and remains in serum as smaller lipoproteins after the degradation of VLDL, we think that the reduction rate of total cholesterol is lower than that of triglyceride. On the other hand, the reduction of free fatty acid in fa/fa rats by M16209 may be due to the reduction of the triglyceride pool in blood and to the increase in the antilipolytic effects of insulin, resulting from the amelioration of insulin resistance in adipose tissues. The peripheral action of M16209, however, is comparatively selective toward muscle and the antilipolytic action is possibly insufficient. Therefore, we think that the rate of reduction of free fatty acid by M16209 is not consistent with the reduction of triglyceride.

It has been demonstrated that the insulin resistance of Zucker fa/fa rats is observed in liver, peripheral tissues including muscle, and white adipose tissue (Terrettaz and Jeanrenaud, 1983). In the present hyperinsulinemic euglycemic clamp study, Zucker fa/fa rats exhibited both reduction in glucose uptake by peripheral tissues and augmentation in hepatic glucose output. Chronic treatment with M16209 normalized the decreased metabolic clearance rate of glucose without affecting hepatic glucose output. Since muscle is among the peripheral tissues principally responsible for insulin-stimulated glucose uptake, it appears that M16209 may stimulate glucose uptake in muscle. This view is consistent with our in vitro finding that M16209 enhanced glucose uptake in L6 muscle cells originally derived from rat skeletal muscle and in isolated rat diaphragm, and that the drug did not affect gluconeogenesis and glycogenolysis in primary cultured rat hepatocytes (unpublished data). Moreover, M16209 increased glycogen synthase-1 activity and fructose-2,6-bisphosphate content in the soleus muscle, and M16209 enhanced the translocation of glucose transporter (GLUT4) from the intracellular pool to the plasma membrane in 3T3-L1 adipocytes (unpublished data). Therefore, we think that the peripheral effects of M16209 are exerted through these mechanisms.

In conclusion, the results of this study indicate that chronic treatment with M16209 ameliorates glucose intolerance, hyperglycemia, hyperlipidemia and hyperinsulinemia in genetically obese and insulin-resistant rodents. These effects of M16209 may be caused by improvement of insulin resistance through enhancement of glucose uptake in muscle.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Miss Michiko Machida and Miss Minako Ban.

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