Lipogenic Action of the Novel Oral Antidiabetic Agent HQL-975 in Genetically Obese Diabetic KK-Ay Mice

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HQL-975 (3-[4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl]-25-propylamino-propionic acid) is a new oral antidiabetic agent which has been shown to be effective in insulin-resistant diabetic animals. In the present study, we examined the effects of HQL-975 on glucose utilization and insulin action in KK-Ay mice with genetically obese non-insulin dependent diabetes (1) Dietary administration of HQL-975 (19 mg/kg/d for 7 d) improved hyperglycemia, hyperlipidemia and hyperinsulinemia in the mice. (2) The HQL-975-treated mice showed enhanced net glucose utilization, that is, glucose was significantly incorporated into total lipids in the white adipose tissue (WAT) and liver, and into glycogen in the diaphragm for the last 24 h of the drug administration period. (3) Treatment improved the decreased stimulatory action of insulin in the epididymal WAT and the agent increased insulin-stimulated lipogenesis from both glucose and acetate. (4) Treatment also increased the activity of lipogenic enzymes such as glycerol-3-phosphate dehydrogenase and fatty acid synthetase. (5) In vitro exposure of WAT to HQL-975 enhanced lipogenesis in the presence of insulin.

From these findings, we conclude that HQL-975 improves glucose utilization of KK-Ay mice through the enhancement of insulin action, which is associated with its lipogenic effects.

Key words HQL-975; non-insulin independent diabetes mellitus; insulin resistance; KK-Ay mice; lipogenesis; adipose tissue

Peripheral insulin resistance is a general feature of patients with non-insulin dependent diabetes mellitus (NIDDM), and is characterized by a reduced responsiveness to insulin and decreased glucose utilization in insulin-target tissues such as adipose tissue and skeletal muscle. The enhancement of insulin action is therefore thought to be an effective pharmacological approach to the treatment of NIDDM.

Insulin, sulfonylurea agents and α-glucosidase inhibitors are clinically used for the treatment of NIDDM. Thiazolidinediones, a new class of antidiabetic agents, are considered insulin-sensitizers, and are expected to reduce insulin resistance in NIDDM and obese patients. The mechanisms of the hypoglycemic and insulin-sensitizing actions of the thiazolidinediones are not yet fully resolved. We recently found a novel oral antidiabetic agent, 3-[4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl]-25-propylamino-propionic acid (HQL-975, Fig. 1). We observed that HQL-975 treatment decreased the plasma glucose level in KK-Ay mice, a rodent model of genetically obese NIDDM, and increased the insulin-stimulated glucose uptake in peripheral tissues; however, it did not affect the plasma glucose and insulin levels in normoglycemic and insulin-deficient diabetic rats. Therefore we suggest that HQL-975 has an insulin-sensitizing action.

We were interested to determine whether HQL-975 might alter glucose utilization as a net response in diabetic KK-Ay mice. In addition, in the present study we investigated the metabolic abnormality and insulin resistance of KK-Ay mice, and the effect of HQL-975 on them. Our studies provide evidence that HQL-975 acts as a lipogenic agent and improves insulin resistance in mice.

MATERIALS AND METHODS

Animals Male KK-Ay/Ta (KK-Ay mice) and normoglycemic ICR mice were obtained from Clea Japan (Osaka, Japan) at the age of 7 weeks. The mice were housed in individual cages in a room controlled for temperature (23 ± 1 °C), humidity (50 ± 4%), and light (12/12 h light-dark cycle, lights on 0800), and were fed laboratory food (CE-2, Clea Japan) and water ad libitum for 3 weeks. The mice used in the experiments were 10 weeks old.

Drug and Reagents HQL-975 was synthesized in our laboratory. Insulin (human, Novolin R40, for the in vivo experiment) was obtained from Novo Nordisk (Copenhagen, Denmark). Bovine serum albumin (BSA, fraction V) was obtained from Sigma (St. Louis, MO). [U-14C]-α-glucose ([14C]-glucose, specific radioactivity: 9.3 GBq/mmol, 251 mCi/mmol) and [1-14C]-acetic acid ([14C]-acetic acid, specific radioactivity: 2.22 GBq/mmol, 60 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Dulbecco's modified minimum essential medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY). Enzyme cofactors were obtained from Oriental Yeast (Tokyo, Japan). The kit reagents used for the determination of plasma parameters were from commercial sources. All other reagents were obtained from Wako Pure Chemical (Osaka).

Hypoglycemic Activity HQL-975 was administered to KK-Ay mice as a 0.01% food admixture in CE-2 powdered food from day 1 to day 8 (for 7 d). The dose was estimated from food intake and body weight to be 19 mg/kg/d. Food was deprived from 0900 to 1300 on day 8, and blood samples taken from tail veins were collected in heparinized hematocrit tubes. The plasma was separated by centrifugation, and the concentrations of glucose, triglyceride, non-esterified fatty acid (NEFA), and insulin were measured using com-

Fig. 1. Chemical Structure of HQL-975

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mmercial kits. In this study, we used untreated normoglycemic ICR mice as the normal control for KK-Ay mice.

Net Glucose Utilization Study HQL-975 was administered to KK-Ay mice as a food admixture (19 mg/kg/d) from day 1 to day 8 (for 7 d). On the morning of day 7, the mice were intraperitoneally injected with 50 μCi/kg 14C-glucose. Twenty-four hours after this injection, the mice were lightly anesthetized by diethyl ether and killed by decapitation. Epididymal, mesenteric and subcutaneous white adipose tissue (WAT), interscapular brown adipose tissue (BAT), liver and diaphragm were excised, and the radioactivities of 14C-glucose incorporated into total lipids (lipogenesis) and into glycerogen (glycogenesis) were then measured as described below.

To assay lipogenesis, 100 mg of the adipose tissue and liver were homogenized in 1 ml of saline, and 2.5 ml of Dole's reagent (isopropanol : n-heptane : 1 N H2SO4 = 40 : 10 : 1) was added and mixed. Next, 1.5 ml of n-heptane and 1 ml of H2O were added and mixed. The radioactivity of the lipids extracted with n-heptane was measured in a liquid scintillation counter type 2500TR (Packard, Meriden, CT) using ACS-II scintillation fluid (Namakai Tesque, Tokyo).

To assay glycerogenesis, 50—100 mg of the epididymal WAT, liver and diaphragm were solubilized in 1 ml of 1 N NaOH at 70 °C, and then carrier glycerogen (2 mg) was added. The glycerogen was precipitated with ice-cold 75% ethanol and washed 4 times with 66% ethanol. The radioactivity of the precipitated glycerogen was measured in a liquid scintillation counter.

In Vivo Insulin-Stimulated Lipogenesis Study in WAT
In this experiment, we examined the effect of HQL-975 on insulin action. Normoglycemic ICR mice, and KK-Ay mice untreated and treated with HQL-975 (19 mg/kg/d, food admixture, for 7 d) were fasted for 24 h. On the morning of day 9, 14C-glucose (50 μCi/kg) or 14C-acetate (100 μCi/kg) with or without insulin (0.1 U/kg) was intraperitoneally injected into the mice. One hour later, the mice were sacrificed, and epididymal WAT was excised. The radioactivities of lipids extracted with n-heptane were measured as described in the section on “Net Glucose Utilization Study.”

Enzyme Activity HQL-975 was given to KK-Ay mice at 19 mg/kg/d for 7 d. On the morning of final administration, epididymal WAT was excised.

To assay glycerol-3-phosphate dehydrogenase (G3PDH), the tissue was homogenized in 25 mm Tris (pH 7.5) containing 1 mm EDTA. The homogenate was centrifuged at 12000×g for 5 min at 4 °C. The infrantasent fraction was used for analysis. G3PDH activity was assayed according to the method of Wise and Green.13

To assay fatty acid synthetase (FAS), the tissue was homogenized in 0.25 m sucrose (pH 7.0) containing 0.5 mm diethanolreitol and 2 mm EDTA. The homogenate was cen-
trifuged at 10500×g for 30 min at 4 °C. The infranant fraction was used for analysis. FAS activity was assayed according to the method of Carey and Dilts.12

In Vivo Lipogenesis in Adipose Tissue Epididymal WAT taken from untreated KK-Ay mice was cut with scissors into small pieces, and washed with saline containing 2% BSA. The incubation medium was composed of DMEM containing 10% heat-inactivated FCS, 100 U/ml penicillin G and 50 μg/ml streptomycin with or without 1 μM/ml insulin. HQL-975 was dissolved in dimethyl sulfoxide (DMSO) and added to the medium. As a control, DMSO was added at a final concentration of 0.1%. The tissue was added to the medium and incubated in a humidified atmosphere under 5% CO2 and 95% air for 18 h at 37 °C. Thereafter, 5 mm 14C-acetate (1 μCi/ml) was added to the medium, followed by incubation for 3 h. The tissue was then washed with saline. Total lipids were extracted with n-heptane, and the radioactivities were measured as described in the section on “Net Glucose Utilization Study.”

Statistical Analysis The data are expressed as the means±S.E.M. Differences were evaluated by Student's t-test for two groups and by Dunnett's multiple comparison test for three or more groups. p values less than 0.05 were considered significant.

RESULTS

Effects of HQL-975 on Levels of Plasma Parameters in Diabetic KK-Ay Mice As shown in Table 1, untreated KK-Ay mice showed significant hyperglycemia, hypertriglyceridermia and hyperinsulinemia compared with the ICR mice. When HQL-975 was administered to KK-Ay mice as a 0.01% food admixture for 7 d, the dose was estimated to be 19 mg/kg/d. The treatment did not alter the food intake (data not shown), whereas it significantly increased final body weight of the mice (44.6±0.6 vs. 41.4±1.3 g, p<0.05). HQL-975 treatment resulted in a significant decrease in the levels of plasma glucose (48%), triglyceride (70%), NEFA (51%) and insulin (77%) in KK-Ay mice (Table 1).

Effect of HQL-975 on Net Glucose Utilization in Epididymal WAT of KK-Ay Mice We examined the effect of HQL-975 on glucose utilization for 24 h as a net response in KK-Ay mice. This net glucose utilization measurement overcomes variations due to circadian changes, but does not allow assessment of hormone actions, i.e. insulin or glucagon effects, on the process. The lipogenesis and glycogenesis in epididymal WAT are shown in Fig. 2. The mice treated with HQL-975 showed a significant increase (60%) in lipogenesis but not in glycogenesis.

Effect of HQL-975 on Insulin-Stimulated Lipogenesis from Glucose in Epididymal WAT of KK-Ay Mice To investigate the insulin action in epididymal WAT of KK-Ay

Table 1. Effects of HQL-975 on Levels of Plasma Glucose, Lipids and Insulin in KK-Ay Mice

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment</th>
<th>Dose (mg/kg/d)</th>
<th>Glucose (mos)</th>
<th>Triglyceride (mos)</th>
<th>NEFA (μmol)</th>
<th>Insulin (μIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR</td>
<td>Untreated</td>
<td>—</td>
<td>181±7</td>
<td>136±10</td>
<td>1312±70</td>
<td>21±3</td>
</tr>
<tr>
<td>KK-Ay</td>
<td>Untreated</td>
<td>—</td>
<td>396±44*</td>
<td>363±10*</td>
<td>930±120</td>
<td>116±7*</td>
</tr>
<tr>
<td>KK-Ay</td>
<td>HQL-975</td>
<td>19</td>
<td>207±6**</td>
<td>109±11**</td>
<td>460±80**</td>
<td>27±6**</td>
</tr>
</tbody>
</table>

HQL-975 was administered as a food admixture for 7 d. After deprivation of food for 4 h on day 8, plasma parameters were determined. Data are the means±S.E.M. (n=5). * p<0.05 vs. ICR untreated, ** p<0.05 vs. KK-Ay untreated.
Fig. 2. Effect of HQL-975 on Net Glucose Utilization in Epididymal WAT of KK-Ay Mice
HQL-975 was administered as a food admixture (19 mg/kg/d) for 7d (from day 1 to day 8), and on the morning of day 7, 14C-glucose (50 µCi/kg) was intraperitoneally given to the mice. On the morning of day 8, the radioactivities of 14C-glucose incorporated into total lipids and glycogen were measured. Data are the means±S.E.M. (n=5). *p<0.05 vs. untreated.

Fig. 3. Effect of HQL-975 on Insulin-Stimulated Lipogenesis from Glucose in Epididymal WAT of KK-Ay Mice
ICR and KK-Ay mice untreated or treated with HQL-975 (19 mg/kg/d for 7d) were fasted for 24h, and then intraperitoneally given 14C-glucose (50 µCi/kg) with or without insulin (0.1 U/kg). At 1 h thereafter, 14C-glucose incorporated into the total lipids was measured. Data are the means±S.E.M. (n=5). *p<0.05 vs. basal, **p<0.05 vs. ICR untreated, ***p<0.05 vs. KK-Ay untreated.

Fig. 4. Effect of HQL-975 on Insulin-Stimulated Lipogenesis from Acetate in Epididymal WAT of KK-Ay Mice
ICR and KK-Ay mice untreated or treated with HQL-975 (19 mg/kg/d for 7d) were fasted for 24h, and then intraperitoneally given 14C-acetate (100 µCi/kg) with or without insulin (0.1 U/kg). At 1 h thereafter, 14C-acetate incorporated into the total lipids was measured. Data are the means±S.E.M. (n=5). *p<0.05 vs. KK-Ay untreated, **p<0.05 vs. basal, ***p<0.05 vs. ICR untreated.

Table 2. Effects of HQL-975 on G3PDH and FAS Activities in Epididymal WAT of KK-Ay Mice

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>Dose (mg/kg/d)</th>
<th>G3PDH (nmol/mg prot/min)</th>
<th>FAS (nmol/mg prot/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR Untreated</td>
<td>—</td>
<td>244±10</td>
<td>1.22±0.34</td>
</tr>
<tr>
<td>KK-Ay Untreated</td>
<td>555±42*</td>
<td>0.44±0.07*</td>
<td>3.85±0.30**</td>
</tr>
<tr>
<td>KK-Ay HQL-975</td>
<td>19</td>
<td>1332±55**</td>
<td>3.85±0.30**</td>
</tr>
</tbody>
</table>

After 7d administration of HQL-975 to KK-Ay mice, G3PDH and FAS activities in epididymal WAT were measured. Data are the means±S.E.M. (n=4). *p<0.05 vs. ICR untreated, **p<0.05 vs. KK-Ay untreated.

pared with the untreated mice. Insulin injection significantly increased lipogenesis in the ICR mice, but it failed to increase in the KK-Ay mice. However, HQL-975 treatment significantly increased insulin-stimulated lipogenesis 3.7-fold in KK-Ay mice. The stimulation by insulin was therefore observed in the treated animals.

Effects of HQL-975 on G3PDH and FAS Activities in Epididymal WAT of KK-Ay Mice After HQL-975 was given to the mice, we measured the lipogenic enzyme activity in epididymal WAT (Table 2). In the WAT of untreated KK-Ay mice, G3PDH activity was increased 2.3-fold, and FAS activity was decreased by 34% compared with the ICR mice. However, HQL-975 treatment significantly increased the activities of both G3PDH and FAS by 2.4-fold and 8.8-fold in KK-Ay mice, respectively.

In Vitro Lipogenic Effect of HQL-975 in Epididymal WAT To examine the in vitro effect of HQL-975, epididymal WAT from untreated KK-Ay mice was incubated with HQL-975 (1, 3, 10 µM) for 18h, and lipogenesis from acetate then measured (Fig. 5). In the absence of insulin, HQL-975 treatment did not alter lipogenesis. Lipogenesis was increased by the addition of 1mM insulin, and further increased by the addition of HQL-975 dose-dependently.

Effect of HQL-975 on Net Glucose Utilization in Other Tissues of KK-Ay Mice We investigated the effect of HQL-975 on the net glucose utilization in mesenteric and subcutaneous WAT, BAT, liver and diaphragm of KK-Ay
mice. HQL-975 treatment significantly increased lipogenesis in mesenteric (2384±350 vs. 663±54 dpm/100 mg/24 h, p<0.05) and subcutaneous WAT (3037±377 vs. 597±38 dpm/100 mg/24 h, p<0.05), respectively. In BAT, the lipogenesis was not altered (4102±366 vs. 3653±430 dpm/100 mg/24 h). The glycogen synthesis in diaphragm was significantly increased in HQL-975-treated mice compared with untreated mice (3368±67 vs. 594±212 dpm/100 mg/24 h, p<0.05). Treatment resulted in a significant increase in lipogenesis (1004±185 vs. 385±37 dpm/100 mg/24 h, p<0.05) but not in glycogen synthesis (2047±596 vs. 2546±390 dpm/100 mg/24 h) in liver.

**DISCUSSION**

This study examined the effect of a new oral agent, HQL-975, on a mice NIDDM model of impaired insulin action. HQL-975 treatment ameliorated the hyperglycemia, hyperlipidemia and hyperinsulinemia in KK-Ay mice. The effective dose of HQL-975 to reduce plasma glucose levels by 30% was five times more potent than pioglitazone.109 Our previous study has suggested that HQL-975 has an insulin-sensitizing effect; the enhancement of insulin-dependent glucose uptake in the muscle and adipose tissue, and the suppression of gluconeogenesis in liver.10,11 In HQL-975-treated KK-Ay mice, lipogenesis was increased as a net response in the epididymal WAT. In contrast, this agent did not alter glycogen synthesis in epididymal WAT. Therefore, we suggest that HQL-975 preferentially enhances the lipogenic pathway rather than the glycogenic one.

Insulin increases lipogenesis from glucose and acetate in adipose tissue41 by the stimulation of glucose uptake and the enhancement of glycolytic and lipogenic enzyme activities. Especially, glucose uptake is closely associated with the rate of lipogenesis. Glucose is transported into adipocytes via the glucose transporter and metabolized to acetyl-CoA and glyceraldehyde 3-phosphate. Acetyl-CoA is incorporated into fatty acids, and then the fatty acids and glyceraldehyde 3-phosphates are esterified. G3PDH and FAS play a role in glyceraldehyde 3-phosphate and fatty acid synthesis, respectively. On the other hand, acetate is converted to acetyl-CoA, and then incorporated into fatty acids followed by esterification.

The present study indicated that insulin action in the epididymal WAT of KK-Ay mice and insulin-stimulated lipogenesis from glucose and acetate were both decreased compared with ICR mice. Furthermore, we observed decreased FAS activity, which might be in part associated with the decreased lipogenesis in the KK-Ay mice. The G3PDH activity, on the contrary, was not decreased. Therefore, we suggest that the fatty acid synthesis pathway is severely deteriorated in the KK-Ay mice, and that the enhancement of this pathway is an effective approach.

In HQL-975-treated KK-Ay mice, the lipogenic action of insulin was improved; this agent enhanced lipogenesis from both acetate and glucose. In the epididymal WAT of treated mice, FAS activity was increased. Furthermore, G3PDH activity was also increased. Our previous findings indicate that the agent increases insulin-stimulated 3H-2-deoxyglucose uptake in the epididymal WAT of KK-Ay mice,107 which suggests that HQL-975 enhances insulin-stimulated glucose transport and/or the following phosphorylation. The in vitro exposure of HQL-975 to epididymal WAT increased lipogenesis in the presence of insulin, whereas it had no effect in the absence of insulin. HQL-975 produced similar metabolic responses in mesenteric and subcutaneous WAT. Thus, it is indicated that WAT is one of the peripheral target tissues of HQL-975, which acts as a lipogenic agent in combination with insulin. We therefore suggest that the improvement of the lipogenic action of insulin by HQL-975 is largely due to enhancement of the fatty acid synthesis pathway. The post-acetyl-CoA pathway might be more important for HQL-975 compared with the pre-acetyl-CoA pathway, since the enhancement of lipogenesis from acetate was higher than that from glucose, especially in the basal state. We also surmise that this action might in part contribute to the hypoglycemic activity of HQL-975, since glucose uptake and subsequent metabolism are obviously increased as a net response.

It is known that fatty acid is secreted from adipocytes to blood as a result of lipolysis and that it largely contributes to the plasma NEFA level. Therefore, the observation that HQL-975 decreases plasma NEFA level suggests that HQL-975 might exert an anti-lipolytic effect in the adipocytes of KK-Ay mice. Plasma triglyceride level, on the contrary, is regulated by hepatic triglyceride synthesis and its secretion to blood. Circulating NEFA is utilized as a substrate for the formation of triglyceride in liver. We surmise that the reduction in the plasma NEFA level might lead to the suppression of hepatic triglyceride production following plasma triglyceride level decrease in the HQL-975-treated mice. In future studies, we will investigate NEFA and triglyceride metabolism in KK-Ay mice compared with ICR mice and the effect of HQL-975 in order to prove the above hypothesis.

Recently, it has been reported that tumor necrosis factor-α (TNFα) is oversecreted from adipocytes and contributes to insulin resistance in obese and NIDDM patients.5,10 However, HQL-975 treatment did not alter the amount of TNFα secreted from epididymal WAT of KK-Ay mice (unpublished observations). Therefore, it is not likely that HQL-975 improves insulin resistance by decreasing TNFα secretion.

In the present study, unfortunately, HQL-975 did not alter net lipogenesis in BAT. However, treated mice showed increased lipogenesis in the insulin-stimulated lipogenesis study (unpublished observations). This result suggests that
HQL-975 potentiates the lipogenesis in BAT although variations due to circadian changes might lead to unchanged net response. The net glycogenesis in diaphragm and net lipogenesis in liver were also stimulated by HQL-975 treatment. These results suggest that the agent increases the energy storage products in KK-Ay mice. Since the plasma NEFA level was decreased in HQL-975-treated KK-Ay mice, it is supposed that NEFA availability is decreased. An increase of plasma NEFA level is known to inhibit insulin-stimulated glucose uptake and to decrease glycogen synthase activity in muscle. Therefore, it is probable that enhanced glucose utilization in diaphragm might be associated with the operation of the glucose-fatty acid cycle.

From these findings, we conclude that HQL-975 acts as a lipogenic agent and that the agent improves the glucose utilization and insulin resistance of KK-Ay mice. We therefore expect that HQL-975 will be an antidiabetic agent for the treatment of insulin resistance in NIDDM patients.

REFERENCES