Actions of the Novel Oral Antidiabetic Agent HQL-975 in Genetically Obese Diabetic db/db Mice

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The hypoglycemic effect of the novel oral agent 3-[4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl]-2S-propylamino-propionic acid (HQL-975) was examined in db/db mice with genetically obese non-insulin dependent diabetes mellitus (NIDDM). The oral administration of HQL-975 at 3.5 and 35.3 mg/kg/d for 7 d decreased the plasma glucose level of these mice in a dose-dependent manner. HQL-975 also significantly decreased the plasma triglyceride, total cholesterol, non-esterified fatty acid and insulin levels. In the oral glucose tolerance test, HQL-975-treated mice showed improved glucose tolerance and decreased endogenous insulin secretion. HQL-975 increased glycemic response to exogenous insulin in the mice. In the HQL-975-treated db/db mice adipocytes, the glucose uptake, insulin binding, and GLUT4 expression were increased compared with those in untreated db/db mice adipocytes. These results indicate that HQL-975 improved insulin action in db/db mice through receptor and post-receptor effects.

In conclusion, HQL-975 is a new oral antidiabetic agent with a hypoglycemic effect which is associated with an insulin-sensitizing effect. This agent may therefore be effective for the treatment of NIDDM.

Key words HQL-975; non-insulin dependent diabetes mellitus (NIDDM); insulin resistance; adipocyte

Non-insulin dependent diabetes mellitus (NIDDM) is characterized by peripheral insulin resistance and impaired insulin secretion, both of which induce metabolic abnormalities, especially in glucose utilization. It is reported that chronic hyperglycemia probably contributes to the microvascular complications of NIDDM of retinopathy, neuropathy and nephropathy,1−3 and also that the hypertriglyceridemia and hyperinsulinemia often observed in NIDDM are risk factors for the microvascular complications of atherosclerosis.4−6 Sulfonylurea agents and α-glucosidase inhibitors are clinically used for the treatment of NIDDM; sulfonylurea agents are known to stimulate insulin secretion,7,8 whereas α-glucosidase inhibitors are known to reduce food-induced elevation of blood glucose.9,10 Thiazolidinediones such as troglitazone and pioglitazone comprise a new class of antidiabetic agents; they are reported to reduce insulin resistance in obese and/or diabetic animals.11,12 Thiazolidinediones are also expected to reduce the risk factors for the microvascular complications of atherosclerosis.13

We recently found that a novel oral antidiabetic agent 3-[4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl]-2S-propylamino-propionic acid (HQL-975, Fig. 1), decreased the plasma glucose level in the genetic NIDDM model KK-Ay mouse.14,15 The genetically obese diabetic db/db mouse is another established model of human NIDDM, and is hyperphagic, hyperlipidemic and hyperinsulinemic. The mouse also shows glucose intolerance and insulin resistance. Insulin resistance in NIDDM is closely associated with insulin receptor downregulation and post-receptor defects.16

It is known that adipocyte is insulin-sensitive, and that the stimulation of glucose uptake is a major insulin action in adipocyte.17 Glucose entry via glucose transporter (GLUT) into a cell is the first and the rate-limiting step of glucose utilization. GLUT1 mainly plays a role in basal glucose uptake, whereas GLUT4 contributes to insulin-stimulated glucose uptake.

In the present study, we investigated the pharmacological effects of HQL-975 on plasma glucose level and insulin action in db/db mice.

MATERIALS AND METHODS

Animals Male C57BL/KsJ-db/db (db/db mice) and ICR mice obtained from Clea Japan (Osaka, Japan) were housed in a room maintained at 23±1°C and 55±5% humidity, with a 12 h light/dark cycle. They were allowed free access to food (CE-2, Clea Japan) and water for 1 week prior to the experiment. Mice used in the experiments were 11 weeks old.

Drug and Reagents HQL-975 was synthesized in our laboratory.14 Bovine serum albumin (BSA, Fraction V), porcine insulin and collagenase (Clostridium histolyticum, type II) were obtained from Sigma (St. Louis, MO, U.S.A.). Block ace (non-fat dried milk) was obtained from Yuki-jirushi (Sapporo, Japan). Rabbit antiserum against the C-terminal peptide of glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) were obtained from East-aces Biologicals (Southbridge, MA, U.S.A.). Dulbecco’s modified minimum essential medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, U.S.A.). 3T3-L1 cells were obtained from Dainippon Pharmaceuticals (Suia, Japan). t[2]S]-Insulin (porcine, specific radioactivity: 81.4 GBq/mmol, 2.2 Ci/mmol), [U-14C]-o-glucose (specific radioactivity: 9.3 GBq/mmol, 251 mCi/mmol) and [1-13C]-acetic acid (specific radioactivity: 2.22 GBq/mmol, 60 mCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). t[3]R]-Protein A (specific activity: 3.46 GBq/mg, 93.4 mCi/mg) was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). All other reagents were obtained from Wako.

Fig. 1. Chemical Structure of HQL-975

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Pure Chemicals (Osaka, Japan).

**Drug Administration and Blood Sampling** HQL-975 was administered daily to db/db mice as a food admixture (0.003 and 0.03%) in CE-2 powdered food from day 1 to day 8. The dosages were estimated from food intake. On the morning of day 8 (in the fed state), blood samples were taken from the tail vein of each mouse and collected in heparinized hematocrit tubes, followed by centrifugation. The concentrations of glucose, triglyceride, non-esterified fatty acid (NEFA), total cholesterol and insulin in the plasma were measured using commercial kits: glucose C-II test, triglyceride-E test, total cholesterol-E test, and NEFA-C test (all four of these from Wako Pure Chemicals), and insulin-seiken-EIA kit (Sanko Junyaku, Tokyo, Japan), respectively.

**Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)** An OGTT and ITT were performed after 7 d of HQL-975 administration. Mice were fasted for 20 h, and then orally received a 2 g/kg glucose solution in the OGTT, or intraperitoneally injected a 0.1 U/kg insulin solution in the ITT. Blood samples were taken at 30, 60 and 120 min thereafter.

**Glucose Uptake in Isolated Adipocytes** HQL-975 was administered to db/db mice for 7 d. On the morning of day 8 (in the fed state), mice were lightly anesthetized by diethyl ether and their epididymal fat pads were removed. Isolated adipocytes were prepared according to the collagenase digestion method of Rodbell [16] and were suspended in Krebs-Ringer HEPES buffer (KRH; 120 mM NaCl, 4.75 mM KCl, 1.2 mM KH2PO4, 10 mM NaHCO3, 1 mM MgSO4, 2.5 mM CaCl2 and 30 mM HEPES, pH 7.4) containing 3% (w/v) BSA.

The glucose uptake study in isolated adipocytes was performed according to the method of Kashiwagi et al. [19] with a slight modification. Adipocytes were incubated in KRH containing 3% (w/v) BSA with the indicated concentration of insulin and 400 nM [U-14C]-glucose for 40 min at 37°C. The reaction was terminated by centrifuging (10000 x g, for 10 s) the cells in a plastic microfuge tube through a layer of dinitrophenyl phthalate (oil floating technique). The radioactivity of the top layer (adipocytes) was measured in a liquid scintillation counter (type 2500TR, Packard, Meriden, CT, U.S.A.) using ACS-II scintillation fluid (Nakalai Tesque, Tokyo, Japan), and the amount of [U-14C]-glucose uptake was calculated. Under these conditions, significant glucose oxidation did not occur. Therefore, production of 14CO2 did not account for the glucose uptake.

**Insulin Binding to Adipocytes** To assay the insulin binding to adipocytes, adipocytes were incubated in KRH containing 3% (w/v) BSA and 10 mM glucose with 2 μCi/ml [125I]-insulin and the indicated concentration of native insulin for 1.5 h at 24°C. The reaction was terminated by the oil floating technique. The radioactivity of the top layer (adipocytes) was measured in a γ-counter (type ARC-2000, Aloka, Tokyo). Specific [125I]-insulin binding was calculated by subtracting the amount of nonspecific binding; the latter was defined as the amount of [125I]-insulin remaining bound in the presence of an excess (200 μg/ml) of native insulin.

**Immunoblotting of GLUT** Immunoblotting of GLUT was performed according to the method of Shimizu et al. [20] with a slight modification. HQL-975 was administered to db/db mice as a food admixture for 7 d. On the morning of day 8 (in the fed state), the epididymal fat pads were removed from the mice and homogenized by a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in 0.25 M sucrose containing 10 mM Tris and 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 13000 x g for 10 min at 4°C, and the infranatants (5, 10, 15 μg protein/spot) were blotted onto a nitrocellulose membrane (Advantec, Tokyo). The blots were blocked with Block ace for 2 h at room temperature, and incubated with anti-GLUT1 or anti-GLUT4 antiserum diluted at 1: 500 with Block ace for 2 h. The membrane was washed 5 times with phosphate-buffered saline (PBS)-T (10 mM sodium phosphate, containing 0.15 M NaCl and 0.1% (v/v) Tween 20, pH 7.4), and then incubated in Block ace containing 0.4 μCi/ml [125I]-protein A for 2 h at room temperature. The membrane was washed 5 times with PBS-T, and the radioactivities of the spots were measured in a γ-counter. The counts of membrane parallelly incubated without blotting of the protein solution were used as a background. The counts were plotted against their respective protein amounts, and the specific counts/μg protein were calculated from the slope of the line. Protein concentrations of the extracts were determined by a protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using BSA as a standard.

**Lipogenesis in 3T3-L1 Adipocytes** 3T3-L1 cells were inoculated into 24-well plates and grown in DMEM containing 10% (v/v) heat-inactivated FCS at 37°C in a humidified atmosphere of 5% CO2–95% air. Confluent cells were staged to differentiate by incubation in the medium containing 0.25 μM dexamethasone (DEX) and 0.5 mM 1-methyl-3-isobutylxanthine (MIX) with or without 10 μg/ml insulin. HQL-975 was dissolved in dimethyl sulfoxide (DMSO), and the solution was diluted 1000-fold in the medium. After 4 d, the cells were washed 3 times with KRH containing 3% (w/v) BSA and 5.5 mM glucose, and incubated in the same buffer containing 5 mM [1-14C]-acetic acid (1 μCi/ml) at 37°C. After 1.5 h, the cells were washed with saline, and Dole's reagent (isopropanol: n-heptane: 1 N H2SO4 = 40: 10: 1) was added and mixed; n-heptane and H2O were then added and mixed. The radioactivities of the lipids extracted with n-heptane were measured in a liquid scintillation counter.

**Statistical Analysis** Data are expressed as the mean ± S.E.M. Differences were evaluated by Student's t-test or Mann-Whitney's U-test for two groups, and by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for three groups. p values less than 0.05 were considered significant.

**RESULTS**

**Effects of HQL-975 on Plasma Metabolic Parameters in db/db Mice** When HQL-975 was administered to diabetic db/db mice as a food admixture at 3.5 and 35.3 mg/kg/d for 7 d, the plasma glucose level was significantly decreased in a dose-dependent manner (p < 0.01 at both dosages, Table 1). Plasma triglycerides, NEFA, total cholesterol and insulin levels were also significantly decreased (p < 0.01 at both dosages). Single oral administration of HQL-975 (10—100 mg/kg), however, did not alter either of the levels of plasma metabolic parameters (data not shown).

**Effects of HQL-975 on Food Intake, Water Intake and
Table 1. Effects of HQL-975 on the Levels of Plasma Metabolic Parameters, Food Intake, Water Intake and Body Weight in db/db Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated</th>
<th>HQL-975 3.5 mg/kg/d</th>
<th>HQL-975 35.3 mg/kg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>488.8±51.7</td>
<td>224.6±37.9**</td>
<td>194.3±15.4**</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>210.3±36.1</td>
<td>63.7±5.2**</td>
<td>40.9±3.1**</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>191.2±6.1</td>
<td>149.2±8.3**</td>
<td>144.8±7.5**</td>
</tr>
<tr>
<td>NEFA (µEq/l)</td>
<td>1133±75</td>
<td>534±52**</td>
<td>300±44**</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>202.2±30.0</td>
<td>77.0±7.9**</td>
<td>55.2±10.9**</td>
</tr>
<tr>
<td>Food intake (g/mouse/d)</td>
<td>5.64</td>
<td>5.68</td>
<td>5.52</td>
</tr>
<tr>
<td>Water intake (g/mouse/d)</td>
<td>6.95</td>
<td>5.07</td>
<td>4.69</td>
</tr>
<tr>
<td>Body weight gain (g/mouse/7d)</td>
<td>0.6±0.5</td>
<td>2.5±1.0*</td>
<td>2.6±0.8*</td>
</tr>
</tbody>
</table>

HQL-975 was administered as a food admixture to mice for 7 d. Data are the mean±SEM; (n=5). *p<0.05, **p<0.01 vs. untreated.

**Body Weight in db/db Mice** Hyperphagia and polydipsia are often observed in diabetic patients and animals. We therefore investigated the effects of HQL-975 on the food intake and water intake of the mice (Table 1), and found that the treatment did not alter the food intake, whereas it decreased the water intake. HQL-975 treatment also significantly increased body weight in the animals (p<0.05 at both dosages, Table 1).

**Effect of HQL-975 on Glucose Tolerance in db/db Mice** In the OGTT, HQL-975 treatment at 35.3 mg/kg/d for 7 d improved hyperglycemia after the glucose load; plasma glucose levels at 30 and 60 min were significantly decreased in the HQL-975-treated mice compared with the untreated mice (Fig. 2A). The treatment significantly decreased the plasma insulin level in the fasting state and at 60 and 120 min (Fig. 2B), and also significantly decreased the plasma NEFA level at 30, 60 and 120 min (Fig. 2C).

![Fig. 2. Effects of HQL-975 on Plasma Glucose (A), Insulin (B) and NEFA (C) Levels in db/db Mice in the OGTT](image)

HQL-975 was administered to the mice as a food admixture for 7 d, and the animals received glucose (2 g/kg, p.o.) on day 8 after 20 h of fasting. Data are the mean±SEM; (n=5). **p<0.01 vs. untreated.

**Effect of HQL-975 on Plasma Glucose Level in ITT** To determine the effect of HQL-975 on the in vivo insulin action in db/db mice, we performed the ITT after 7 d treatment of HQL-975 at 35.3 mg/kg/d (Fig. 3). Insulin decreased the plasma glucose level from the 60-min point in the untreated mice, whereas it decreased it from the 30-min point in the HQL-975-treated mice. Plasma glucose level at 30 min in the treated mice was significantly decreased compared with that in the untreated mice (p<0.05).

**Effect of HQL-975 on Glucose Uptake in db/db Mice Adipocytes** In this experiment, we used normoglycemic ICR mice as a normal control of db/db mice. An insulin-dependent glucose uptake study was performed in isolated...
adipocytes, and the results are shown in Fig. 4. In the ICR mice adipocytes, insulin increased the glucose uptake in a dose-dependent manner. In the db/db mice adipocytes, the basal and insulin-stimulated glucose uptakes were significantly decreased compared with those in the ICR mice adipocytes. However, HQL-975 (35.3 mg/kg/d for 7 d) treatment significantly increased the basal and insulin-stimulated glucose uptakes in db/db mice. The half maximal response to insulin occurred at 2.9 ng/ml in the ICR mice, at 5.0 ng/ml in the untreated db/db mice and at 5.0 ng/ml in the HQL-975-treated db/db mice.

**Effect of HQL-975 on Insulin Binding in db/db Mice**

**Adipocytes** The insulin binding in the db/db mice adipocytes was significantly decreased compared with that in the ICR mice adipocytes (Fig. 5). The Scatchard analysis revealed that the insulin receptor number in the db/db mice adipocytes was decreased to ∼20% compared with that in the ICR mice adipocytes, whereas the affinity was not changed (data not shown). HQL-975 (35.3 mg/kg/d for 7 d) significantly increased the insulin binding in the db/db mice adipocytes (Fig. 5). Scatchard analysis showed that the insulin receptor number in the HQL-975-treated db/db mice adipocytes was increased ∼2-fold compared with that in the untreated db/db mice adipocytes, whereas the affinity was not changed (data not shown).

**Effect of HQL-975 on GLUT Expression in db/db Mice** As shown in Fig. 6, we investigated the amounts of 2 types of GLUT expressed in adipose tissues. HQL-975 was administered to db/db mice at 35.3 mg/kg/d for 7 d. No sig-
significant difference was observed in GLUT1 expression among the normoglycemic ICR mice, HQL-975-treated and untreated db/db mice (Fig. 6A). The insulin-regulatable GLUT4 expression in the db/db mice was significantly decreased compared with that in the ICR mice (p<0.01, Fig. 6B). However, HQL-975 slightly but significantly increased the GLUT4 expression in db/db mice (p<0.05).

In Vitro Effect of HQL-975 on the Lipogenic Action of Insulin in 3T3-L1 Adipocytes Since isolated adipocytes are fragile in long-term in vitro incubation, we used 3T3-L1 adipocytes in this experiment. Lipogenesis concomitant with adipocyte differentiation in 3T3-L1 cells was increased by the treatment of DEX plus MIX, and was further increased by the addition of insulin. HQL-975 treatment promoted insulin action; it did not alter the lipogenesis in the absence or presence of DEX plus MIX, while it significantly increased the lipogenesis in the presence of DEX, MIX plus insulin (p<0.01 at both dosages).

DISCUSSION

NIDDM is a complicated chronic disorder, characterized by peripheral insulin resistance and pancreas β-cell abnormality. Diabetic db/db mice show obesity, hyperglycemia, glucose intolerance, hyperlipidemia and hyperinsulinemia, and are an excellent model of NIDDM. In this study, we examined the effects of HQL-975 on the plasma glucose level and insulin action in db/db mice. HQL-975 exerted a hypoglycemic effect when administered repeatedly as a food admixture. In KK-Ay mice, another model of NIDDM, HQL-975 was more potent than pioglitazone or metformin, a biguanide agent; the effective dosage to reduce plasma glucose level by 25% occurred at 3 mg/kg/d in the HQL-975-treated (14,15) at 6 mg/kg/d in the pioglitazone-treated, (12) and at >100 mg/kg/d in the metformin-treated mice (unpublished data). The plasma triglyceride, total cholesterol, NEFA and insulin levels of the db/db mice were also decreased by the treatment with HQL-975. From these results, we surmise that HQL-975 not only ameliorates hyperglycemia but also reduces risk factors of atherosclerosis.

Since HQL-975 did not affect the food intake of the mice, it is clear that its hypoglycemic and hypolipidemic effects were not due to a decrease in food intake. In a previous study, we found that HQL-975 had no effect on the plasma glucose and insulin levels in normoglycemic and insulin-deficient streptozotocin rats, (13) and that it did not affect intestinal sucrose, maltase or isomaltase activities in vitro or in vivo (unpublished data). In the present study, however, HQL-975 improved the glucose tolerance of db/db mice despite decreasing insulin secretion during OGTT. Furthermore, HQL-975 accelerated glycemic response to exogenous insulin in db/db mice during ITT. Taken together, these suggest that HQL-975 has an insulin-sensitizing effect, but it does not have an insulin-like, a sulfonylurea agent-like or an α-glucosidase inhibitor-like effect.

Insulin resistance in NIDDM is associated with insulin receptor downregulation and post-receptor defects. (16) The present study indicated that the abnormality of stimulative insulin action on the glucose uptake in the db/db mice adipocytes was at least associated with the receptor downregulation and decrease of GLUT4 expression. HQL-975 ameliorated the stimulative insulin action on glucose uptake in these adipocytes, and preferentially improved insulin-responsivity rather than insulin-sensitivity. The amelioration by HQL-975 was indicated to be associated with the upregulation of insulin receptor and the increase of GLUT4 in db/db mice. Since insulin receptor downregulation is closely associated with hyperinsulinemia, its upregulation by HQL-975 may be largely due to the amelioration of hyperinsulinemia. No significant difference was observed between GLUT1 expression in the untreated and HQL-975-treated mice. Unfortunately, this result did not explain the difference in the basal glucose uptake of the two groups. On the contrary, the GLUT4 expression in the HQL-975-treated db/db mice was significantly increased compared with that in the untreated mice. Therefore, the improvement of insulin-stimulated glucose uptake, in part, due to the restoration of GLUT4 expression. These results suggest that HQL-975 improves the insulin action through receptor and post-receptor effects in db/db mice. It is reported that the increase in the plasma NEFA level inhibits insulin-regulated glucose uptake, but the precise mechanism is still unknown. (21) In the present study, HQL-975 decreased the plasma NEFA level in the non-fasting state and after glucose load. Therefore, we also suggest that the decrease of NEFA level in the HQL-975-treated db/db mice may have, in part, contributed to the improvement of glucose uptake.

We found that in vitro HQL-975 treatment enhanced the lipogenic action of insulin during the adipocyte differentiation of 3T3-L1 cells. This finding suggests that HQL-975 directly exerts an insulin-sensitizing effect and acts as a lipogenic agent, increasing glucose utilization and the capacity of glucose and lipid storage.

In the present study, HQL-975 decreased the plasma insulin level of db/db mice in the non-fasting state and during OGTT. The precise mechanism is still undetermined. However, we suppose that the hypoglycemic effect of HQL-975 might lead to the improvement of hyperinsulinemia, since HQL-975 did not alter the insulin level in normoglycemic rats in which the plasma glucose level was not affected by HQL-975. (15)
In summary, HQL-975 showed significant hypoglycemic and insulin-sensitizing effects in diabetic db/db mice. Its insulin-sensitizing effect was closely associated with insulin receptor and post-receptor effects. HQL-975 also improved the hyperlipidemia and hyperinsulinemia in mice, and consequently, we expect that it will be effective for the treatment of NIDDM.

REFERENCES