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Combination therapy with PPARγ and PPARα agonists increases glucose-stimulated insulin secretion in db/db mice

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Submitted 8 April 2002; accepted in final form 10 December 2002

Yajima, Ken, Hiroshi Hirose, Haruhisa Fujita, Yoshiko Seto, Hiroshi Fujita, Kaname Ukeda, Kiichi Miyashita, Toshihide Kawai, Yukihiro Yamamoto, Takeo Ogawa, Taketo Yamada, and Takao Saruta. Combination therapy with PPARγ and PPARα agonists increases glucose-stimulated insulin secretion in db/db mice. Am J Physiol Endocrinol Metab 284: E966–E971, 2003; 10.1152/ajpendo.00149.2002.—Although peroxisome proliferator-activated receptor (PPAR)γ agonists ameliorate insulin resistance, they sometimes cause body weight gain, and the effect on insulin secretion is unclear. We evaluated the effects of combination therapy with a PPARγ agonist, pioglitazone, and a PPARα agonist, bezafibrate, and a dual agonist, KRP-297, in male C57BL/6J mice and db/db mice, and we investigated glucose-stimulated insulin secretion (GSIS) by in situ pancreatic perfusion. Body weight gain in db/db mice was less with KRP-297 treatment than with pioglitazone or pioglitazone + bezafibrate treatment. Plasma glucose, insulin, triglyceride, and nonesterified fatty acid levels were elevated in untreated db/db mice compared with untreated C57BL/6J mice, and these parameters were significantly ameliorated in the PPARγ agonist-treated groups. Also, PPARγ agonists ameliorated the diminished GSIS and insulin content, and they preserved insulin and GLUT2 staining in db/db mice. GSIS was further increased by PPARγ and α agonists. We conclude that combination therapy with PPARγ and PPARα agonists may be more useful with respect to body weight and pancreatic GSIS in type 2 diabetes with obesity.

peroxisome proliferator-activated receptor; glucose-stimulated insulin secretion; glucolipotoxicity; insulin resistance; type 2 diabetes with obesity

MATERIALS AND METHODS

Materials. Pioglitazone, bezafibrate, and KRP-297 were donated by Takeda (Osaka, Japan), Kissei (Matsumoto, Japan), and Kyorin Pharmaceuticals (Tokyo, Japan). Pioglitazone, P + B, and KRP-297 were each blended with CE-2 by use of 0.1% carboxymethylcellulose.

Animals. Five-week-old male C57BL/6J mice and male db/db mice were purchased from Japan Clea. Our institution’s guidelines for the care and use of laboratory animals were followed. Both mouse strains were fed CE-2 control chow and water ad libitum for 1 wk and each of the following...
drug-blended chow and water ad libitum for the following 4 wk: PIO [C57BL/6J: 0.02% (wt/wt), 31.3 ± 0.3 mg·kg⁻¹·day⁻¹; db/db: 0.02% (wt/wt), 34.2 ± 0.4 mg·kg⁻¹·day⁻¹], P + B [C57BL/6J: 0.02% (wt/wt), 29.2 ± 0.5 mg·kg⁻¹·day⁻¹; db/db: 0.02% (wt/wt), 33.8 ± 0.4 mg·kg⁻¹·day⁻¹] + [C57BL/6J: 0.06% (wt/wt), 97.3 ± 1.5 mg·kg⁻¹·day⁻¹; db/db: 0.07% (wt/wt), 111.0 ± 1.5 mg·kg⁻¹·day⁻¹], and KRP-297 [C57BL/6J: 0.02% (wt/wt), 30.2 ± 0.4 mg·kg⁻¹·day⁻¹; db/db: 0.02% (wt/wt), 32.2 ± 0.4 mg·kg⁻¹·day⁻¹]. Food intake, water intake, and body weight of all mice were measured in the morning 3 to 4 times/wk. Mice were fasted overnight and underwent operation for in situ pancreatic perfusion.

Measurement of metabolic parameters in plasma. Blood from the retroorbital sinus of 10-wk-old mice in a nonfasting state was collected into hemotocrit tubes coated with EDTA. Plasma glucose and triglycerides were determined with a Fuji Dry-Chem 5500 (Fuji Film, Tokyo, Japan). Plasma nonesterified fatty acid (NEFA) concentration was determined by the NEFA-C test (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin concentration was determined by enzyme immunoassay (EIA; Morinaga Institute of Biological Science, Yokohama, Japan).

Pancreatic insulin content. Each pancreatic specimen was homogenized in 1.8 ml of acid ethanol. The homogenates were stored at 4°C for 48 h and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were pooled, and the pellets were then resuspended in 0.2 ml of acid ethanol. After centrifugation, the supernatants were diluted 1:10,000 in 0.1 M PBS containing 0.25% BSA. Insulin concentration was determined by EIA.

In situ pancreatic perfusion. The mice were anesthetized with 50 mg/kg pentobarbital sodium after an overnight fast. The celiac artery and portal vein were cannulated after other vessels had been ligated. Nonrecirculating perfusion was begun at a constant flow rate of 0.5 ml/min. The perfusion medium consisted of Krebs-Ringer bicarbonate buffer containing 3.0% (wt/vol) dextran T-40 (Pharmacia, Uppsala, Sweden), 1.0% (wt/vol) BSA (fraction V, RIA grade; Sigma Chemical, St. Louis, MO), and 20 mM HEPES. A 95% O₂–5% CO₂ gas mixture was bubbled through the perfusate, and pH was maintained between 7.35 and 7.45. The pancreatic venous effluent was collected into tubes via a cathether in the portal vein at 2-min intervals, frozen immediately, and stored at −20°C for subsequent EIA. To achieve postoperative equilibration, the pancreas was perfused for 15 min with a buffer containing the glucose concentration of the initial 10-min experimental period (5.6 mM). Thereafter, insulin response to a high-glucose concentration (16.7 mM) was examined for 20 min, and glucose concentration was then returned to 5.6 mM for another 20 min.

Histopathological examination. Pancreata from mice in all groups that were not operated on for in situ pancreatic perfusion were fixed in 35–38% Formalin for histopathological studies. The pancreata were embedded in paraffin, sectioned, and then stained with hematoxylin and eosin (H + E). Immunohistochemical studies were also performed using anti-human insulin guinea pig antibodies (Oriental Yeast, Osaka, Japan) and rabbit anti-GLUT2 polyclonal antibody (Chemicon International, Temecula, CA).

Statistical analyses. All results were expressed as means ± SE. Statistical analyses were performed with the Statview program (version 5.0-J, SAS Institute, Cary, NC). Analysis of variance (ANOVA), followed by a post hoc Bonferroni-Dunn multiple comparison test, was used to evaluate differences among groups. A P value <0.05 was considered statistically significant.

RESULTS

Body weight change, total food intake, and total water intake. The body weight of animals in PPARγ agonist-treated groups increased more than that of animals in the untreated group in both mouse strains (Table 1). Body weight gain in the KRP-297-treated group was less than that in other treated groups in db/db mice, but not in C57BL/6J mice. Total food intake in the PPARγ agonist-treated groups was also significantly greater than that in the untreated group in C57BL/6J mice. On the contrary, total food intake of the KRP-297-treated group was the smallest in db/db mice. The PPARγ agonist-treated groups in C57BL/6J mice showed a mild increase in total water intake, whereas the untreated group in db/db mice showed a marked increase.

Metabolic parameters in plasma. Plasma glucose, insulin, triglyceride, and NEFA levels were elevated in untreated db/db mice compared with untreated C57BL/6J mice, and these parameters were significantly ameliorated in the PPARγ agonist-treated groups (Fig. 1).

Pancreatic insulin content. Pancreatic insulin content of untreated db/db mice was almost completely diminished compared with untreated C57BL/6J mice (Fig. 2). Pancreatic insulin content of the PIO- and P + B-treated db/db mice was ameliorated compared with untreated db/db mice, although these differences were not statistically significant (P = 0.051 by ANOVA).

Table 1. Body weight change, total food intake, and total water intake during 4 wk in C57BL/6J and db/db mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Body Weight Change, g</th>
<th>Total Food Intake, g</th>
<th>Total Water Intake, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>Untreated</td>
<td>3.06 ± 0.25</td>
<td>96.35 ± 1.59</td>
<td>158.45 ± 5.12</td>
</tr>
<tr>
<td></td>
<td>PIO</td>
<td>4.91 ± 0.17*</td>
<td>112.29 ± 1.38*</td>
<td>189.88 ± 7.95*</td>
</tr>
<tr>
<td></td>
<td>P + B</td>
<td>4.12 ± 0.34*</td>
<td>108.27 ± 1.75*</td>
<td>178.79 ± 6.33</td>
</tr>
<tr>
<td></td>
<td>KRP-297</td>
<td>4.70 ± 0.18*</td>
<td>107.98 ± 1.71*</td>
<td>198.33 ± 7.63*</td>
</tr>
<tr>
<td>db/db</td>
<td>Untreated</td>
<td>9.90 ± 0.46</td>
<td>191.50 ± 2.77</td>
<td>505.60 ± 17.64</td>
</tr>
<tr>
<td></td>
<td>PIO</td>
<td>21.17 ± 0.45*</td>
<td>182.80 ± 3.07</td>
<td>196.10 ± 5.12*</td>
</tr>
<tr>
<td></td>
<td>P + B</td>
<td>19.30 ± 0.48*</td>
<td>180.67 ± 3.29</td>
<td>197.40 ± 8.23*</td>
</tr>
<tr>
<td></td>
<td>KRP-297</td>
<td>15.46 ± 0.43*†</td>
<td>164.93 ± 2.04*‡‡</td>
<td>191.13 ± 4.31*</td>
</tr>
</tbody>
</table>

Values are means ± SE. PIO, pioglitazone; P + B, PIO and bezafibrate. *P < 0.0083 vs. untreated group in each strain; †P < 0.0083 vs. PIO group in each strain; ‡‡P < 0.0083 vs. P + B group in each strain.
The pancreatic insulin content of the KRP-297-treated \textit{db/db} mice was almost the same as that of untreated C57BL/6J mice.

\textit{In situ pancreatic perfusion.} There was no difference in GSIS among the four groups of C57BL/6J mice (Fig. 3A). In \textit{db/db} mice (Fig. 3B), whereas GSIS in the untreated group was diminished compared with that in C57BL/6J mice, that in the PPAR\textgamma agonist-treated groups was preserved compared with that in the untreated group. GSIS in the PIO-treated group showed no significant difference from that in the untreated group in \textit{db/db} mice, whereas GSIS in both the P + B- and the KRP-297-treated groups was significantly increased (Table 2).

\textit{Histopathological findings.} The islets of untreated \textit{db/db} mice showed hyperplastic changes (Fig. 4). Insulin in these islets of untreated \textit{db/db} mice was extremely reduced, and the staining pattern was scattered. Islets in the PIO- and P + B-treated \textit{db/db} mice showed hyperplasia and almost normal staining of insulin. In islets of the KRP-297-treated \textit{db/db} mice,

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Fig. 1. Plasma glucose (A), insulin (B), triglyceride (C), and nonesterified fatty acid (NEFA, D) levels at 10 wk for untreated groups [solid bars, \(n = 5\), C57BL/6J mice shown as reference (C57), and open bars, \(n = 5\), (\textit{db/db})]; pioglitazone (PIO)-treated group [vertically striped bars, \(n = 5\) (\textit{db/db})]; combined pioglitazone- and bezafibrate (P + B)-treated group [checked bars, \(n = 5\) (\textit{db/db})]; and KRP-297-treated group [hatched bars, \(n = 5\), (\textit{db/db})]. Each value represents a mean \(\pm\) SE. \(P < 0.0083\) vs. untreated \textit{db/db} mice.

Fig. 2. Pancreatic insulin content at 10 wk for untreated group [solid bar, \(n = 3\) (C57), and open bar, \(n = 5\) (\textit{db/db})]; PIO-treated group [vertically striped bars, \(n = 8\) (\textit{db/db})]; P + B-treated group [checked bars, \(n = 8\) (\textit{db/db})]; and KRP-treated group [hatched bars, \(n = 5\) (\textit{db/db})]. Data of untreated C57BL/6J are shown for reference. Each value represents a mean \(\pm\) SE. \(P = 0.051\) by ANOVA.

Fig. 3. Glucose-stimulated insulin secretion (GSIS) by in situ pancreatic perfusion in C57BL/6J mice (A) and \textit{db/db} mice (B): untreated groups (\(\bullet\), \(n = 6\) for both C57 and \textit{db/db}) ; PIO-treated groups (\(\circ\), \(n = 6\) for both C57 and \textit{db/db}) ; P + B-treated groups (\(\triangledown\), \(n = 6\) (C57) and 5 (\textit{db/db}) ) ; KRP-treated groups (\(\star\), \(n = 6\) for both C57 and \textit{db/db}). Each value represents a mean \(\pm\) SE (A). Each value of untreated, PIO-treated, and KRP-treated groups represents a mean \(\pm\) SE (B).
the hyperplasia was improved, and the staining of insulin was almost normal. The results of the insulin staining were consistent with those of pancreatic insulin content. GLUT2 was expressed on the /H9252>-cell membrane of normal C57BL/6J mice. Although GLUT2 expression was not detected in untreated db/db mice, it was preserved in PPAR /H9253 agonist-treated db/db mice as well as in C57BL/6J mice. However, there was no significant difference in GLUT2 expression among the PIO-, P /H11001 B-, and KRP-297-treated groups of the db/db mice.

DISCUSSION

PPARγ agonists are well suited for the treatment of type 2 diabetic patients with obesity who have severe insulin resistance. However, because PPARγ is a master regulator of adipocytes (22), its activation may induce increased fat mass and obesity. The degree of body weight gain is dependent on different PPARγ agonists (18). Also, body weight gain by PPARγ agonists is reported not only in rodents (11, 27) but also in humans (16, 33), and it is speculated to be due to adipogenesis by the drug effect (8, 31, 35) as well as increased food intake (4, 40, 41). However, the effect of PPAR agonists on insulin secretion is unclear.

In the present study, body weight in the PPARγ agonist-treated groups increased significantly with increased food intake in C57BL/6J mice. However, weight gain in db/db mice was less with KRP-297 treatment compared with PIO or P /H11001 B treatment. We speculate that this is due to the effects of a dual agonist that enhances lipid catabolism and reduces fat mass by PPARγ activation, as previously suggested (2, 5a). We consider that KRP-297 ameliorated obesity most, so that pancreatic insulin content of the KRP-297-treated group was almost the same as that in normal mice (26), and the islets of the KRP-297-treated group were not

<table>
<thead>
<tr>
<th>GSIS, mU</th>
<th>C57BL/6J</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–10 min</td>
<td>10–30 min</td>
</tr>
<tr>
<td>Untreated</td>
<td>79.02 ± 52.01</td>
<td>1,301.10 ± 333.07</td>
</tr>
<tr>
<td>PIO</td>
<td>151.80 ± 27.54</td>
<td>1,857.53 ± 269.23</td>
</tr>
<tr>
<td>P + B</td>
<td>138.32 ± 22.75</td>
<td>1,637.92 ± 194.65</td>
</tr>
<tr>
<td>KRP-297</td>
<td>29.35 ± 19.08</td>
<td>1,556.51 ± 202.71</td>
</tr>
</tbody>
</table>

Values are means ± SE. GSIS, glucose-stimulated insulin secretion. *P < 0.0083 vs. untreated group in each strain during the same period; †P < 0.0083 vs. PIO group in each strain during the same period; ‡P < 0.0083 vs. KRP-297 group in each strain during the same period.

Fig. 4. Histopathological examination of pancreas from untreated C57BL/6J mice and db/db mice untreated and treated with PIO, P + B, and KRP. H & E, hematoxylin and eosin staining; Insulin, anti-insulin antibody staining; GLUT2, anti-GLUT2 antibody staining. Original magnification: all ×80.
hyperplastic in contrast to those of the PIO- or P + B-treated groups.

Also, we have shown in this study that PPARγ agonists normalized the elevated plasma glucose, insulin, triglyceride, and NEFA levels in db/db mice. However, we did not find an additional effect of a PPARα agonist on these parameters. Plasma glucose level in the KRP-297-treated group was the lowest in db/db mice, probably because of the lowest food consumption in addition to the drug effect.

GSIS in C57BL/6J mice showed a good response and was not changed by 4-wk treatment with PPARα and/or PPARγ agonists. In untreated db/db mice, on the other hand, there was no GSIS response, due to insulin shortage resulting from severe insulin resistance, glucotoxicity, and lipotoxicity. Plasma insulin level in untreated db/db mice at this age, however, was higher than that in any other group; the islets of untreated db/db mice secreted insulin against an extremely high plasma glucose level. The stimulatory glucose level of the perfusate was 16.7 mM in our experiments, which was lower than the plasma level of untreated db/db mice. Therefore, we speculate that the islets of untreated db/db mice might be desensitized to glucose and thus did not show a GSIS response at 16.7 mM. However, we have shown reduced insulin staining with hyperplasia of islets, and pancreatic insulin content was barely detectable in untreated db/db mice, both suggesting insulin shortage.

In the present study, GSIS in db/db mice was preserved by PPARγ agonist treatment. There was amelioration of plasma glucose, insulin, triglycerides, and NEFA levels in these groups. We also observed preserved insulin staining in the islets of the PPARγ agonist-treated groups, and we concluded that the insulin-saving effects of PPARγ agonists were mediated by ameliorating insulin resistance. De Souza et al. (3) also suggested amelioration of insulin resistance in Zucker diabetic fatty (ZDF) rats. These authors showed that there was no direct effect of pioglitazone on the pancreas in perfusion experiments. Although P + B and KRP-297 treatments in our study significantly increased GSIS in db/db mice, it is unclear why GSIS was increased by the addition of a PPARα agonist. Because there was no difference in plasma glucose, insulin, triglyceride, and NEFA levels among the PIO-, P + B-, and KRP-297-treated groups, increased insulin secretion in the P + B- and KRP-297-treated groups might not be through amelioration of glucolipotoxicity but through increased glucose sensitivity in pancreatic β-cells.

Lipotoxicity may cause β-cell abnormalities, loss of GSIS and GLUT2, and triglyceride accumulation (37, 38). Higa et al. (9), using ZDF rats, reported that troglitazone prevented these features of lipotoxicity. Because the peroxisome proliferator response element (PPRE) was identified in the rat GLUT2 gene (promoter) (17), PPARα and/or PPARγ agonists may affect PPRE in the GLUT2 gene in pancreatic β-cells. Wang et al. (39) suggested that PPARα might be one of the transcription factors involved in the direct upregulation of GLUT2 in normal rat islets. Furthermore, Zhou et al. (42) reported that expression of PPARα was suppressed in ZDF rat islets. In the present study, GSIS was increased by addition of a PPARα agonist, and we evaluated the staining of GLUT2, which is associated with glucose sensing and GSIS. We showed that treatment with PPARγ agonist preserved GLUT2 in db/db mice. However, it was difficult to identify differences in GLUT2 staining among the PIO-, P + B-, and KRP-297-treated groups. It is possible that a PPARα agonist reduced the islet triglyceride content in db/db mice, although we were not able to examine this. Further studies will be needed to clarify the direct effect of PPARα on islet function.

To summarize, body weight in PPARγ agonist-treated groups increased significantly with increased food intake in C57BL/6J mice. In contrast, weight gain in db/db mice was less with KRP-297 treatment compared with PIO- or P + B treatment. PPARγ agonists ameliorated the diminished GSIS by improving insulin sensitivity in db/db mice, and GSIS was further increased by the combined PPARγ and PPARα agonists. We conclude that combination therapy with PPARγ and PPARα agonists may be more useful than PPARγ alone with respect to body weight gain and pancreatic GSIS in type 2 diabetes with obesity.

REFERENCES


There were two errors. First, units of plasma insulin in Figure 1B were incorrectly shown as mUl/l. They should be mU/l. Second, symbols in Figure 3, A and B, were published incorrectly. The correct Figure 3 is printed here, with the original legend.

**Fig. 3.** Glucose stimulated insulin secretion (GSIS) by in situ pancreatic perfusion in C57BL/6J mice (A) and db/db mice (B): untreated groups (●, n = 6 for both C57 and db/db); PIO-treated groups (○, n = 6 for both C57 and db/db); P+B-treated groups ([, n = 6 (C57) and 5(db/db)]; KRP-treated groups („, n = 6 for both C57 and db/db). Each value represents mean ± SE (A). Each value of untreated, PIO-treated, and KRP-treated groups represents a mean – SE (B). Each value of P+B-treated group represents a mean + SE (B).