Insulin sensitizer YM268 ameliorates insulin resistance by normalizing the decreased content of GLUT4 in adipose tissue of obese Zucker rats

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Abstract
Genetically obese Zucker rats exhibit mild hyperglycaemia and hyperinsulinaemia suggesting the existence of peripheral insulin resistance. We have examined the effects of YM268, an analogue of thiazolidinedione, on the content and translocation of a glucose transporter (GLUT4) in epididymal adipose tissue in 11-week-old obese and lean Zucker rats. The administration of YM268 at a dose of 10 mg/kg for 2 weeks ameliorated hyperglycaemia, hyperinsulinaemia, and impaired glucose tolerance after glucose load in obese rats. The GLUT4 content per fat pad in obese rats was reduced to 36% of that in lean littermates. Obese rats treated with YM268 increased GLUT4 concentrations in their fat pads from a basal value of 36% up to 191% of the level in lean rats. Furthermore, in adipocytes prepared from obese rats, an increase in the ratio of GLUT4 in plasma membrane to the total amount of GLUT4 (PM-GLUT4 ratio) induced by the submaximal concentration of insulin (0.3 nmol/l) was significantly attenuated compared with that in lean rats. But the maximum effect of insulin (3 nmol/l) was not attenuated. Meanwhile, YM268 had no significant effect on the attenuated PM-GLUT4 ratio in response to insulin in obese rats. These data suggested that one of the mechanisms by which YM268 improved insulin resistance in obese Zucker rats was to normalize the decreased GLUT4 content in the adipose tissue.

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Introduction
It is well known that the mild hyperglycaemia and hyperinsulinaemia observed in genetically obese Zucker (fa/fa) rats is primarily due to insulin resistance (1, 2), which is characterized by both peripheral (skeletal muscle and adipose tissue) and hepatic insulin resistance. Earlier work indicated that adipocytes from young obese Zucker rats exhibited insulin-induced hyper-responsiveness of glucose uptake (3, 4), but the glucose uptake increased by insulin gradually declined as a function of age (4). Such alterations in insulin-stimulated glucose uptake in adipocytes may be explained by changes in glucose transporter levels in these cells. Recent studies on the cellular mechanism of glucose uptake have revealed that the entry of glucose into cells is facilitated by glucose transporter proteins. One such protein GLUT4 is expressed in only adipose tissue, muscle and heart tissue in which glucose uptake is regulated by insulin (5, 6). Insulin stimulates cellular glucose uptake by mediating the translocation of intracellular GLUT4 to the plasma membrane (7). In an attempt to understand the metabolic disorder in obese Zucker rats, it is important to investigate whether the insulin resistance observed in these animals is due to a decreased GLUT4 content, a decreased GLUT4 translocation in response to insulin, or both. King et al. (8) showed that insulin resistance in obese Zucker rats is associated with a failure of GLUT4 translocation to the plasma membrane, but it is not associated with changes in the GLUT4 content of the plasma membrane in muscle. Conversely, Galante et al. (9) reported that insulin resistance in the skeletal muscle of obese Zucker rats is not associated with lack of GLUT4 translocation. There is still considerable controversy about this genetic disorder of glucose metabolism in obese Zucker rats.

Thiazolidinedione derivatives have been reported to improve insulin sensitivity in animal models showing insulin resistance (10, 11). Since insulin resistance is often an important factor in the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM), these agents may be used to prevent or treat NIDDM. Troglitazone and darglitazone increased the effectiveness of insulin in NIDDM patients (12, 13). In this study we examined the efficacy and mode of action of YM268, an analogue of thiazolidinedione, on the insulin resistance observed in obese Zucker rats. The aim of this study was to examine the relationship between the insulin resistance observed in obese Zucker rats and the GLUT4 content, as well as GLUT4 translocation from...
intracellular pools to the plasma membrane in adipose tissue. The second purpose of this study was to determine the effects of YM268 on the GLUT4 content and GLUT4 translocation stimulated by insulin.

Materials and methods

Materials

All reagents used in this study were of analytical grade and are commercially available. YM268 (bis {4-[(2,4-dioxo-5-thiazolidinyl) methyl] phenyl} methane) was synthesized at Yamanouchi Pharmaceutical Co. Ltd (Tsukuba, Japan).

Animals and experimental design

Forty-eight male Zucker obese (fa/fa) rats and sixty of their lean littermates (Fa/-) were purchased from Charles River Laboratories (Wilmington, MA, USA) at 7 weeks of age and kept in a 12 h light:12 h darkness cycle. They were allowed free access to a standard pellet diet. YM268 (10 mg/kg) was given orally to Zucker rats at 9 weeks of age for 14 days. A glucose tolerance test was performed on day 11 after overnight fasting and all rats were killed on day 15. Total particulate membranes or isolated adipocytes were prepared from their epididymal and perirenal adipose tissues.

Blood sampling and analytical methods

Blood samples were obtained from the tail vein using heparinized haematocrit tubes. Glucose and insulin concentrations were determined by a glucose oxidase method and RIA using rat insulin as a standard.

Preparation of total particulate membranes from adipose tissue

The membrane fraction from epididymal adipose tissue was prepared according to the method described by Oka et al. (14) with a minor modification. Briefly, epididymal adipose tissue isolated from six rats were pooled and homogenized in a Potter–Elvehjem glass–Teflon homogenizer in 20 mmol/l Hepes, 250 mmol/l sucrose and 1 mmol/l EDTA, pH 7.4. The homogenates were centrifuged at 900 g av for 10 min. The resulting supernatant was centrifuged at 146 000 g av for 10 min. The supernatant (S-2) mentioned above was centrifuged at 147 000 g av for 75 min. The sucrose solution was drained from the bottom of the centrifuge tube and divided into 17 fractions (each 0.75 ml) according to the original method (16, 17). Fractions 3–7 and 13–17 were reported to be PM and GO fractions respectively and were separately pooled. 5'-Nucleotidase (a plasma membrane marker) activity and protein concentration were measured. Fractions 3–7 showed much higher activity of 5'-nucleotidase than fractions 13–17. These findings were supported by measurement of protein concentration because fractions 13–17 had no significant protein peak. Fractions 3–7 were diluted with buffer A and centrifuged at 147 000 g max for 60 min. The pellet was saved.

In order to obtain the GO fraction in pellet form, the supernatant (S-2) mentioned above was centrifuged at 32 000 g max for 30 min and the resulting supernatant was centrifuged again at 147 000 g max for 60 min. The total activity of 5'-AMPase in the GO fraction obtained by this method was seven or eight times lower than in the PM fraction in obese or lean rats. These values were comparable with those reported by Kono et al. (19) using the original subcellular fractionation method.

Immunoblot analyses of glucose transporters

Protein concentrations in isolated membranes were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as a standard. The membrane proteins were incubated with SDS reducing buffer (2% SDS, 10% glycerol and 100 mmol/l dithiothreitol in 62.5 mmol/l Tris buffer, pH 6.8) for 1 h at room temperature and subjected to SDS-PAGE on a 10%
polyacrylamide gel according to the method described by Laemmli (20). The proteins resolved were then electrophoretically transferred to polyvinylidene difluoride membrane filters (Bio-Rad Japan, Tokyo, Japan) at 180 mA for 1 h in a blotting cell (TEFCO, Nagano, Japan) as described by Towbin et al. (21). The filters were washed with PBS containing 0.05% Tween 20, blocked with Block-Ace (Snow Brand, Sapporo, Japan) for 2 h, and then incubated overnight with polyclonal antibodies specific for the C-terminus of rat GLUT4 (East Acres Biologicals, Southbridge, MA, USA). The filters were washed and then incubated for 2 h with horseradish peroxidase-conjugated protein A (Amersham Japan, Tokyo, Japan) and the labelled bands were visualized using ECL-chemiluminescence detection reagents (Amersham Japan), and scanned by densitometry using a flying-spot scanner CS-9000 (Shimadzu, Kyoto, Japan) at a wavelength of 600 nm. For the quantitative measurement of GLUT4, a linear regression between the intensity of chemiluminescence and the amount of membrane protein (log (μg/lane)) loaded into the gel was used as a calibration curve. The calibration curve was drawn by least-squares fitting. Since the calibration curve did not pass through zero, differing concentrations of membranes proteins (2, 4, 8 and 16 μg/lane) from the adipose tissue of lean Zucker rats were run on each gel as standards in order to minimize the effect of non-specific binding of the antibodies. The intra- and interassay variation in the amount of GLUT4, shown by immunoblotting, were 7.5% or 10.8% respectively when 5 μg membrane protein was loaded into a gel.

Data presentation

We calculated the total amount of GLUT4 per mg membrane protein, and the total amount of GLUT4 per fat pad. To assess the insulin-induced GLUT4 translocation from the GO to the PM fractions in isolated adipocytes, the ratio of GLUT4 in the PM fraction to the total amount of GLUT4 (PM-GLUT4 ratio) was expressed as follows: PM-GLUT4 ratio (%) = PM-GLUT4 × 100/(PM-GLUT4 + GO-GLUT4).

YM268 did not significantly change the total GLUT4 content (PM-GLUT4 + GO-GLUT4) in adipocytes from obese or lean rats during 15 min of incubation with insulin, since the total GLUT4 content in the adipocytes in the presence of 0, 0.3 or 3.0 nmol/l insulin was 1:1.08:1.27 (obese rats) and 1:1.05:1.16 (lean rats) respectively, based on their relative chemiluminescence intensities.

Statistical analyses

Comparisons between experimental groups were made using the Mann–Whitney U test, t-test, one-way ANOVA, or Dunnett’s multiple range test. Differences were accepted as significant at the P < 0.05 level.

Results

Effects of YM268 on the plasma concentrations of glucose and insulin in obese Zucker rats

It is well known that mild hyperglycaemia and hyperinsulinaemia in genetically obese Zucker rats is due to insulin resistance (1, 2). YM268 at a dose of 10 mg/kg was given orally for 14 days to 9-week-old obese or lean Zucker rats. Plasma glucose and insulin concentrations in obese rats were decreased by days 7 and 14 (Fig. 1). In lean rats, however, plasma insulin
levels but not glucose levels were lowered (data not shown). This agent had no effect on body weight in obese and lean rats (Table 1).

**Effects of YM268 on glucose tolerance and insulin secretion in obese Zucker rats**

Glucose intolerance and hypersecretion of insulin after glucose load were observed in obese Zucker rats (Fig. 2). YM268 improved glucose tolerance and normalized not only the basal concentration of insulin in the fasted state, but also the hypersecretion of insulin after glucose load. Thus it seems that YM268, like other thiazolidinedione derivatives reported to be insulin sensitizers (10), ameliorated insulin resistance in genetically obese rats.

**Effect of YM268 on GLUT4 levels in the adipose tissue of obese and lean Zucker rats**

To elucidate the mechanism(s) by which YM268 ameliorates insulin resistance we examined GLUT4 levels in the epididymal adipose tissues (fat pad) of obese and lean Zucker rats. As shown in Table 1, YM268 increased the weight of the fat pads by 25% and the total amount of membrane protein per fat pad by 241% in obese Zucker rats. Total membrane protein was also increased in lean rats. In order to minimize the effect of the difference in tissue weights and total membrane protein levels in the fat pads among the groups, GLUT4 levels were expressed per mg membrane protein or per fat pad (see Materials and methods). The amount of GLUT4 in crude membrane extracted from adipose tissues was quantified by immunoblotting using specific antisera. When an equal amount of membrane protein was applied to the gel, GLUT4 per mg membrane protein in adipose tissue of obese Zucker rats was about 30% of the levels observed in lean control rats, and was increased up to 48% of the control level by YM268 treatment (Fig. 3A and B). The effects of YM268 on GLUT4 were remarkable when GLUT4 levels were shown on a per fat pad basis. YM268 increased GLUT4 levels from 36% to 191% of the levels observed in the lean controls (Fig. 3C). YM268 also increased GLUT4 levels in lean Zucker rats to 256% (per mg

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**Table 1** Effects of YM268 (10 mg/kg) or vehicle given orally for 14 days on the body weight, epididymal adipose tissue weight, and total amount of membrane protein in lean (Fa/–) and obese (fa/fa) Zucker rats. Values are means ± S.E.M. of six rats.

<table>
<thead>
<tr>
<th></th>
<th>Lean (Fa/–)</th>
<th>Obese (fa/fa)</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>YM268</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>301 ± 15.5</td>
<td>292 ± 8.9</td>
</tr>
<tr>
<td>Adipose tissue weight (g)</td>
<td>2.3 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Total membrane protein</td>
<td>Per fat pad (mg)</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Per gram tissue (mg)</td>
<td>0.24 ± 0.01</td>
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* Difference between YM268-treated and YM268-untreated groups is significantly different at P < 0.05 (t-test).
There is considerable evidence showing that insulin increases glucose transport activity in the PM fraction, while it decreases this activity in the GO or low-density microsomal fractions (22, 23). To investigate the possible relationship between the insulin resistance observed in obese Zucker rats and translocation of glucose transport activity in the plasma membrane, the GLUT4 content of the PM and GO fractions (Fig. 5), and the ratio between GLUT4 in the plasma membrane and the total GLUT4 content (PM-GLUT4 ratio) in isolated adipocytes from epididymal tissue was examined (Table 2). As shown in Fig. 5, insulin increased GLUT4 levels in the PM whereas it decreased GLUT4 levels in the GO fractions from adipocytes of lean Zucker rats. Furthermore, insulin at 0.3 nmol/l significantly increased the PM-GLUT4 ratio from the basal value without insulin in adipocytes from lean rats (Table 2), but insulin at the same concentration failed to increase it in those from obese rats. The maximum effect of insulin was not attenuated in obese rats since a ten times higher concentration of insulin (3 nmol/l) increased the PM-GLUT4 ratio from the basal value without insulin in adipocytes from lean rats (Table 2), but insulin at the same concentration failed to increase it in those from obese rats. These findings may suggest that GLUT4 translocation in response to submaximal concentrations of insulin is decreased in obese rats. YM268, however, had no significant effect on GLUT4 translocation in response to insulin in lean and obese rats.

**Discussion**

In this study we examined the relationship between insulin resistance and the content and translocation of GLUT4 in adipose tissue of genetically obese Zucker rats. The GLUT4 content in the adipose tissue of 11-week-old obese Zucker rats was about one-third of that in lean littermates (Fig. 3). Furthermore, the attenuation of GLUT4 translocation in response to submaximal concentrations of insulin was also observed in adipocytes from obese rats (Table 2). Obese Zucker rats treated with YM268 had increased GLUT4 contents from a basal value of 30% of the value observed in the lean littermates to 48% on a per mg protein basis, or from 36% to 191% on a per fat pad basis (Fig. 3). These data suggest that one of the mechanisms by which YM268 ameliorates peripheral insulin resistance is an increase in the GLUT4 content of adipose tissue in genetically obese rats. The effect of YM268 on the GLUT4 content expressed on a per fat pad basis was much greater than that expressed on a per mg protein basis. These differences may arise from increases in the total concentrations of membrane proteins (3.4-fold), as...
well as the increased weight of adipose tissue (1.3-fold) (Table 1). The increases in GLUT4 concentrations induced by YM268 were masked by increased concentrations of other membrane proteins when expressed on a per mg protein basis. Thus the GLUT4 protein content was expressed as per mg membrane protein or per fat pad in this study.

Although it is well known that the insulin resistance observed in the aged, obese rats (24), in rats fed high-fat/low-carbohydrate diets (25), and in streptozocin diabetic rats (26) results from the relative depletion of GLUT4 in the intracellular pool of adipocytes, the mechanisms of insulin resistance observed in obese Zucker rats is still controversial. Hainault et al. (3) reported that GLUT4 levels in the adipocytes of obese Zucker rats were 4.5 times higher than in the lean littermates on a per cell basis at 30 days of age. Pedersen et al. (4), however, clearly showed age-related changes in the amount of GLUT4 protein per adipocyte in obese Zucker rats; namely, the GLUT4 contents in the adipocytes of these animals were increased 3.5 and 2.5 times the levels observed in lean littermates at 5 and 10 weeks of age respectively. But the GLUT4 content in the 20-week-old obese rats was significantly lower than in the lean animals. Using data obtained from several obese and diabetic animal models, Klip et al. (27) statistically analyzed the relationships between GLUT4 protein or mRNA contents in adipose tissue and blood glucose and insulin levels. They found that high levels of circulating glucose depressed GLUT4 mRNA levels in adipose tissue and the amount of GLUT4 protein in muscle. These findings may suggest that the development of hyperglycaemia with age in obese Zucker rats is one of the dominant factors in determining tissue GLUT4 mRNA and protein levels.

Using the method for isolation of the PM and GO fractions which was proposed by Kono et al. (16, 17), we compared GLUT4 translocation in response to insulin in adipocytes from obese or lean Zucker rats treated with or without YM268. Contrary to the results obtained from lean rats, insulin at 0.3 nmol/l failed to increase the PM-GLUT4 ratio in adipocytes from obese rats (Table 2). However, the maximum effect of insulin was not attenuated in obese rats. These findings may

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### Table 2  Effects of YM268 on the insulin-dependent translocation of GLUT4 and adipocytes isolated from lean and obese Zucker rats. The ratio of GLUT4 in the plasma membrane to the total GLUT4 content in adipocytes isolated from lean and obese Zucker rats treated with or without YM268 is shown. Results are the means ± S.E.M. from three separate experiments in obese rats or four in lean rats. Adipocytes pooled from six animals were used in each experiment.

<table>
<thead>
<tr>
<th>Insulin (nmol/l)</th>
<th>Lean (Fa/¹)</th>
<th>Obese (Fa/fa)</th>
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<tbody>
<tr>
<td></td>
<td>No YM268</td>
<td>YM268</td>
</tr>
<tr>
<td>0.0</td>
<td>13.0 ± 2.4</td>
<td>7.1 ± 1.6</td>
</tr>
<tr>
<td>0.3</td>
<td>37.0 ± 2.0**</td>
<td>41.0 ± 1.6**</td>
</tr>
<tr>
<td>3.0</td>
<td>49.5 ± 0.4**</td>
<td>46.2 ± 0.4</td>
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The effect of insulin is significantly different (* P < 0.05, ** P < 0.01) from the corresponding controls without insulin (one-way ANOVA, Dunnett’s multiple range test).
suggest that the sensitivity of GLUT4 translocation to submaximal concentrations of insulin is decreased in obese rats. YM268 had no effect on the insulin-induced increases in the PM-GLUT4 ratio in obese rats. One of the most important findings in our study was that the sensitivity of GLUT4 translocation to submaximal concentrations of insulin in obese Zucker rats was diminished. YM268 did not improve its translocation defect in response to insulin. These findings are partly compatible with a recent report (28) showing that an insulin sensitizer, BRL 49653, caused a 2.5-fold increase in the total GLUT4 content and a 2.6-fold increase in plasma membrane-associated GLUT4 in adipocytes from C57BL/6 obese (ob/ob) mice. Pioglitazone, however, failed to correct the defect in glucose transport and glucose transporter protein translocation in skeletal muscle of the obese Zucker rat (29), suggesting that this sensitizer had no significant effect on GLUT4 translocation in response to insulin.

Although the mechanisms by which thiazolidinedione derivatives improve insulin resistance are not entirely clear, a wide variety of mechanisms have already been proposed. First, pioglitazone and troglitazone might reduce insulin resistance by increasing phosphorylation of the insulin receptors. It has been demonstrated that pioglitazone increased the insulin-stimulated autophosphorylation of insulin receptors by activating kinases of the receptor in muscle of genetically Wistar fatty rats (30) and rats fed high fat (31). Second, pioglitazone also increased reduced GLUT4 mRNA content in genetically obese KKAy mice and streptozocin-induced diabetic rats (32). Finally, in cultured fibroblasts expressing human insulin receptors, troglitazone increased the autophosphorylation of the insulin receptor which was decreased by high concentrations of glucose in the medium (33). The mechanism underlying the desensitization of insulin receptors induced by hyperglycaemia is not understood precisely, but troglitazone may reduce the phosphorylation of the serine and threonine residues of the z subunit of the insulin receptor by inhibiting the activity of protein kinase C. The phosphorylation sites might be responsible for the inactivation of kinase activity of the insulin receptors (34). It is highly likely that these insulin sensitizers ameliorated insulin resistance by facilitating insulin’s signal transduction. If the previously mentioned mechanism can be applied to YM268, one would expect that YM268 increases GLUT4 content indirectly by altering insulin’s signal transduction pathway. We are now investigating this possibility.

In contrast to the marked hypoglycaemic action of YM268 in obese Zucker rats, YM268 like other insulin sensitizers did not cause hypoglycaemia in lean Zucker rats (35). However, immunoblot analyses in this study revealed that the GLUT4 content of adipose tissue in lean Zucker rats was much higher than in obese rats after YM268 treatment. The increases in the GLUT4 translocation ratio induced by insulin were similar in both lean and obese rats treated with YM268. These facts indicate that the GLUT4 content of the plasma membrane is much higher in lean rats than it is in obese rats. If the GLUT4 content in adipocytes is the only determinant factor for blood glucose concentrations, we still do not have enough evidence to explain this observation. This remains to be proven, along with the effects of YM268 on GLUT4 regulation in normal animals.

In summary, we have examined the relationship between insulin resistance and the GLUT4 content and GLUT4 translocation to the plasma membrane in response to insulin in adipose tissue from obese Zucker rats. YM268 ameliorated insulin resistance in part by normalizing the decreased content of GLUT4 in obese Zucker rats.

Acknowledgements
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