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Actions of novel antidiabetic thiazolidinedione, T-174, in animal models of non-insulin-dependent diabetes mellitus (NIDDM) and in cultured muscle cells

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1 The antihyperglycaemic effect and the possible mechanism of action of T-174, a novel thiazolidinedione derivative, was determined *in vivo* and *in vitro*.

2 Oral administration of T-174 markedly improved hyperglycaemia, hyperinsulinaemia, hyperlipidaemia, and glucose intolerance in genetically obese and diabetic yellow KK (KK-A^y) mice (0.2–15.5 mg kg^{-1} day^{-1}, for 7 days) and Zucker fatty rats (1.4–11.4 mg kg^{-1} day^{-1}, for 6 days). The ED_{50} values for the glucose lowering action of T-174 and pioglitazone, another thiazolidinedione antidiabetic, were 1.8 and 29 mg kg^{-1} day^{-1}, respectively in KK-A^y mice; T-174 was about 16 times more potent than pioglitazone.

3 The hypoglycaemic effect of exogenous insulin in KK-A^y mice was enhanced after the administration of T-174. A hyperinsulinaemic euglycaemic clamp study in Zucker fatty rats showed an amelioration of whole-body insulin resistance by the T-174 treatment.

4 Insulin-stimulated glucose metabolism was enhanced in adipocytes from KK-A^y mice treated with T-174. The insulin receptor number of the adipocytes was increased without a change in the affinity of the receptor.

5 The hypomagnesaemia in KK-A^y mice was completely restored by T-174.

6 In cultured L6 myotubes, glucose consumption and [^{3}H]-2-deoxy-glucose transport were enhanced by T-174 (EC_{50}; 6 and 4 μM, respectively). Combination of insulin with T-174 was additive to stimulate glucose disposal.

7 These results suggest that the antihyperglycaemic effect of T-174 was mediated by enhanced insulin action. This was associated with amelioration of the hypomagnesaemia and T-174 directly increased basal and insulin-stimulated glucose utilization by cultured muscle cells.

Keywords: T-174; thiazolidinedione; antidiabetic agents; insulin sensitivity; magnesium; L6 cell line; skeletal muscle

Introduction

Insulin resistance is a common feature of almost all patients with non-insulin-dependent (type 2) diabetes mellitus (NIDDM) (DeFronzo *et al*., 1992). The presumed central role of insulin resistance suggests that enhanced insulin action may be a preferred pharmaceutical therapy for NIDDM. Thiazolidinedione derivatives (TZDs), like troglitazone, pioglitazone, and BRL 49653, comprise a new class of orally active antidiabetic agents that enhance insulin action in animal models of NIDDM and NIDDM patients (Saltiel & Olefsky, 1996). We have also identified a new potent agent of this structural class, T-174 (5-[[2-(2-naphthalenylmethyl)-5-benzoxazolyl]-methyl]-2,4-thiazolidinedione (Figure 1)), which improved glycaemic control in an animal model of NIDDM when administered chronically (Arakawa *et al*., 1997).

T-174, like other TZDs, is a ligand for a specific subclass of nuclear receptors, peroxisome proliferator-activated receptor γ (PPARγ), which is abundantly present in adipose tissues (Lehmann *et al*., 1995; Mizukami & Taniguchi, 1997). Stimulation of PPARγ promotes differentiation of preadipocytes to adipocytes *in vitro* (Forman *et al*., 1995) and a PPARγ-dependent mechanism has been postulated for the antidiabetic action of TZDs (Willson *et al*., 1996). In contrast, a recent study has shown that troglitazone improved insulin sensitivity independent of its effect on adipose tissues (Burant *et al*., 1997). Therefore, the role of non-adipose mechanisms of TZDs has yet to be explored. Hypomagnesaemia is commonly found in patients with diabetes mellitus (Paolisso *et al*., 1990) and increasing evidence suggests a linkage between magnesium deficiency and insulin resistance (Paolisso *et al*., 1989; Balon *et al*., 1994, 1995; Suárez *et al*., 1995). Pioglitazone has been demonstrated to increase intracellular magnesium concentrations *in vitro* (Nadler & Scott, 1994). In addition, evidences suggest that TZDs can enhance glucose disposal directly at the level of skeletal muscle tissues (El-Kebbi *et al*., 1994; Ciaraldi *et al*., 1995; Fürnsinn *et al*., 1997), which is the major site of insulin resistance (Baron *et al*., 1988, 1991; Garvey, 1992).

In the present study, we have investigated the effect of T-174 on the insulin-resistant syndrome in genetically obese diabetic yellow KK (KK-A^y) mice and obese glucose intolerant Zucker fatty rats. We have also determined the effects of T-174 on the plasma magnesium homeostasis *in vivo* and the direct effect on glucose handling by L6 skeletal muscle cells.

Methods

Animals

Male KK-A^y mice (5–10 weeks old) were obtained from CLEA Japan (Tokyo, Japan). Age-matched normal male ddY

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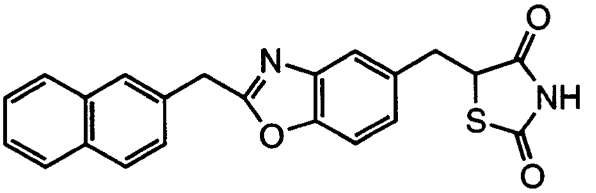

Figure 1 Structure of T-174.

mice (Japan SLC, Shizuoka, Japan) were used for comparison. They were housed individually in plastic cages with bedding. Zucker fatty (*fa*/*fa*) rats and their lean littermates (*FA*/?) were purchased from Charles River (Raleigh, NC, U.S.A.) at the age of 5–8 weeks. Two or three rats were housed per metal cage. All animals were maintained with a commercial diet, CE-2 (CLEA Japan), consisting of 52.7% carbohydrate, 23.6% protein, 4.4% fat, 4.9% fiber, 6.6% minerals and vitamins. Food and water were provided *ad libitum* before and during the experiments. The animal rooms were controlled for temperature (23 ± 2°C), humidity (55 ± 5%) and light (12 h light-dark cycle). All experiments were started after at least one week of acclimation period. The animals were divided into experimental groups matched for both initial body weights and blood glucose levels.

Drug administration and blood sampling

Drugs were administered as dietary admixtures in CE-2 powdered diet for 6–7 days. The doses were estimated from the daily diet intakes and body weights.

Blood samples for determining glucose concentrations were obtained from tail tip and then deproteinized with $Ba(OH)_2$ and $ZnSO_4$. After centrifugation, the glucose concentration in the supernatant was determined. To prepare the plasma for assay of insulin, triglycerides (TG), nonesterified fatty acids (NEFA), and magnesium levels, the blood was collected in heparinized hematocrit tubes and centrifuged.

Insulin and glucose tolerance tests

In the insulin tolerance test, mice were fasted for 20 h and received i.p. injection of human insulin (Actrapid®) at a dose of 0.5 u kg^{-1}. In the oral glucose tolerance test (OGTT), both rats and mice were given a 20% glucose solution (2 g glucose kg^{-1}) after 20 h of fasting.

Glucose metabolism and insulin binding in isolated adipocytes

Adipocytes were obtained from the epididymal fat pads of KK-A^y mice by digestion with collagenase according to the method of Rodbell (1964) with the exceptions that collagenase was used at a concentration of 6 mg g^{-1} tissue and both preparation and incubation buffers were 30 mM N-[2-Hydroxyethyl]piperazinne-N′-[2-ethanesulphonic acid] (HEPES)-buffered Krebs-Ringer bicarbonate solution (pH 7.4; 120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 1.2 mM KH_2PO_4, 10 mM $NaHCO_3$) containing 2% bovine serum albumin (BSA). Adipocyte counting was performed using a microscope with a Bürker-Türk counting chamber and the cell diameters were measured with a micrometer.

To measure the rate of glucose oxidation, 2×10^5 cells were incubated in 1 ml of buffer containing 0.2 mM glucose, 0.5 μCi [1-^{14}C]glucose, and 0–25 ng ml^{-1} porcine insulin at 37°C for 120 min. Hyamine solution® was used to trap $^{14}CO_2$ evolved and the radioactivity was measured. Lipogenesis from glucose was estimated by determining the incorporation of ^{14}C into total lipids, which were extracted with hexane as described by Rodbell (1964).

Insulin binding was measured by incubation of the adipocytes (2×10^5 cells) in 0.5 ml of buffer with 0.5 ng ml^{-1} of ^{125}I-insulin and various concentrations of unlabeled insulin at 24°C for 60 min. The cells were separated from the medium by the oil floatation method (Gliemann *et al*., 1972) and the radioactivity was counted. The specific binding was calculated by subtracting the non-specific binding, which was determined in the presence of excess unlabeled insulin (200 μg ml^{-1}), from the total binding.

Hyperinsulinaemic euglycaemic clamp experiment

Overnight fasted rats were surgically prepared for the hyperinsulinaemic euglycaemic clamp study under anesthesia with pentobarbital sodium (50 mg kg^{-1}, i.p.), as described by Kraegen *et al*. (1983). In brief, an intravenous catheter (PE20, Cray Adams, Parsippany, NJ, U.S.A.) was inserted into the left jugular vein (for blood sampling) and filled with heparin-saline (50 iu ml^{-1} 0.9% saline). Two other catheters were fitted with 27-gauge needles, and inserted into left and right femoral veins for exogenous glucose and insulin infusion, respectively. All experiments were conducted at least 30 min after the surgery to allow the plasma glucose level elevated by the surgical stress to return to the basal fasting level.

Human insulin (Actrapid®) infusion was started at time zero at a rate of 28 μ kg^{-1} min^{-1} and maintained at a constant rate throughout the study. Blood was sampled at 5 min intervals and immediately analysed for the glucose concentrations. The plasma glucose level was then maintained at the basal fasting level by infusion of appropriate amount of 10% glucose solution. Following 1 h stabilization period, the glucose infusion rate during the 2nd h (GIR_{60-120}) was determined. Additional blood samples were collected for the determination of plasma insulin levels at 0, 60, 80, 100 and 120 min.

Glucose disposal and 2-deoxy-glucose uptake in cultured L6 myotubes

The rat skeletal muscle cell line, L6, was obtained from the American Type Tissue Culture Collection (Rockville, MD, U.S.A.). The myoblasts were initially grown in Dulbecco's modified Eagle's medium containing 25 mM glucose, 60 μg ml^{-1} kanamycin sulfate supplemented with 10% foetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2. After confluence, differentiation of the cells into myotubes was induced by changing the supplementation of the medium from 10% to 2% FCS. Cells were maintained for 13–15 days under these cultural conditions, allowing greater than 90% of the cells to become multinucleated myotubes.

Treatment of the cells was initiated by replacing the medium with agents and/or porcine insulin. Agents were dissolved in dimethyl sulphoxide (DMSO) and added at the indicated concentrations. Control cells were treated with a matching concentration of DMSO. For glucose utilization studies, glucose disappearing from the culture medium in 96-well plate by 48 h, which was determined by measurement of glucose concentrations, was considered to be taken up and consumed by the cells. Glucose uptake was measured 48 h after treatment using [^{3}H]-2-deoxy-glucose as described earlier (Hayes *et al*., 1993). Briefly, cells grown in 24-well plate were washed three times with HEPES-buffered Krebs-Ringer phosphate solution supplemented with 0.1% BSA, pH 7.4, and transport of 10 μM [^{3}H]-2-deoxy-glucose (0.5 μCi ml^{-1} well^{-1}) was measured at 37°C for 10 min in the same buffer. The assay was terminated by aspiration and washing cells with ice-cold PBS. Samples were extracted with 1% SDS and counted in a scintillation counter. Non-specific transport was defined as that which occurred in the presence of 10 μM cytochalasin B.

Analytical methods

Blood glucose levels and glucose concentrations of the culture media were determined using commercially available kits based on the glucose oxidase method (New Blood Sugar Test, Boehringer Mannheim, Mannheim, Germany and Glucose B-test Wako, Wako Pure Chemical Industries, Osaka, Japan). During the euglycaemic clamp experiment, plasma glucose levels were measured immediately (within 1 min after sampling) using a glucose analyzer (APEC, Inc., Danvers, MA, U.S.A.). Plasma immunoreactive insulin was measured by a radioimmunoassay kit (Insulin kit 'Eiken', Eiken Chemical, Tokyo, Japan) with human insulin as a standard. Plasma TG and NEFA were determined by enzymatic assays using kits supplied by Eiken Chemical (Tokyo, Japan). The plasma magnesium levels were measured by Magnesium B-test Wako (Wako Pure Chemical Industries, Osaka, Japan). A preliminary study showed the high correlation ($r = 0.99$) between the results from the Magnesium B-test Wako and atomic absorption spectrophotometry. Protein was determined with the BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.).

Materials

T-174 and pioglitazone were synthesized at the Lead Optimization Research Laboratory of Tanabe Seiyaku Co., Ltd. (Saitama, Japan). Human insulin (Actrapid®) was purchased from Novo Nordisk (Bagsvaerd, Denmark), porcine monocomponent insulin from Sigma (St. Louis, MO, U.S.A.), porcine ^{125}I-Insulin from New England Nuclear (Boston, MA, U.S.A.), [1-^{14}C]glucose and [^{3}H]-2-deoxy-glucose from Amersham (Amersham, U.K.), collagenase, type I from Worthington (Freehold, NJ, U.S.A.), Hyamine solution® from Packard (Downers Grove, IL, U.S.A.), BSA, Fraction V from Intergen (Purchase, N.Y., U.S.A.). FCS (JRH Bioscience) was supplied by Nichirei (Tokyo, Japan). All other chemicals used were of reagent grade or of tissue culture grade.

Statistics

The data are expressed as the mean ± s.e. mean. For comparison of two groups, *P*-values were calculated by two-tailed unpaired Student's *t*-test. Multiple comparisons were performed by Dunnett's method, except as otherwise described in the legend. In all cases, $P < 0.05$ was considered to be statistically significant. Calculation of ED$_{50}$ and EC$_{50}$ values was performed by a nonlinear least squares analysis using a four-parameter logistic model. ED$_{50}$ and EC$_{50}$ values were defined as half-maximal effective doses and concentrations, respectively.

Results

Antidiabetic activity of T-174 in KK-A^y mice and Zucker fatty rats

Administration of T-174 (0.2 to 15.5 mg kg^{-1} day^{-1}) for 7 days lowered blood glucose levels in KK-A^y mice in a dose-dependent manner (Figure 2). A significant decrease in blood glucose levels was observed at a dose as low as 0.6 mg kg^{-1} day^{-1} of T-174; the ED$_{50}$ value was 1.8 mg kg^{-1} day^{-1} and the maximum decrease was achieved with approximately 5 mg kg^{-1} day^{-1}. Pioglitazone also caused a dose-dependent fall in blood glucose (ED$_{50}$; 29 mg kg^{-1} day^{-1}), but it was less potent than T-174. When the ED$_{50}$ values for the hypoglycaemic effects were compared, T-174 was about 16 times more

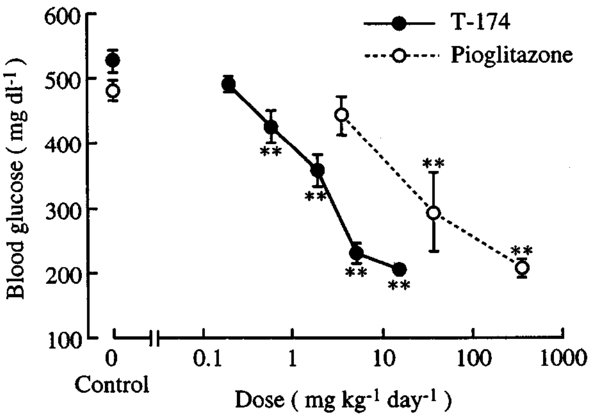

Figure 2 Effects of T-174 and pioglitazone on blood glucose levels in KK-A^y mice. T-174 or pioglitazone was administered to 12-week-old male KK-A^y mice for 7 days. Each symbol represents the mean and vertical lines show s.e.mean of five to six animals. **$P < 0.01$ compared with respective control.

Table 1 Effects of T-174 on body weight, food intake, plasma insulin and plasma lipid levels in KK-A^y mice

Animal groups	*Dose* (mg kg^{-1} day^{-1})	*Body weight* (g)	*Food intake* (g day^{-1})	*Plasma insulin* (μu ml^{-1})	*Plasma TG* (mg dl^{-1})	*Plasma NEFA* (μEq l^{-1})
Control	–	42.2±0.3	8.3±0.3	1120±78	821±85	454±46
T-174	0.2	42.5±0.7	8.1±0.3	1567±269	746±98	399±40
	0.6	43.5±0.4	8.3±0.4	1137±314	550±59*	291±36**
	1.9	46.2±0.8**	8.5±0.3	670±195	310±49**	165±18**
	5.1	46.6±1.3**	7.5±0.2	423±189	157±13**	134±6**
	15.5	47.2±1.0**	7.7±0.2	153±32**	138±11**	137±13**

T-174 was given to 12-week-old male KK-A^y mice, weighing 38.9–45.2*g* (average: 42.1*g*), for 7 days. Each value represents as the mean±s.e.mean of six animals. *$P < 0.05$ and **$P < 0.01$ compared with control. Abbreviations: TG, triglycerides; NEFA, non-esterified fatty acids.

potent than pioglitazone. Table 1 shows a dose-dependent decrease in plasma insulin, TG and NEFA levels as well as the blood glucose concentration. Although there was no significant effect on the food intake, the body weights increased significantly. Similarly, in Zucker fatty rats given T-174 (1.4 and 4.5 mg kg^{-1} day^{-1}, for 6 days), blood glucose, plasma insulin, TG and NEFA levels were significantly decreased, but there was no significant change in body weight (Table 2).

After oral administration of T-174 for 7 days, both mice and rats were fasted overnight and subjected to an OGTT. As shown in Figure 3, there was an elevated blood glucose level and hypersecretion of insulin in response to an oral glucose load in the untreated Zucker fatty rats, compared with their lean littermates. The impaired glucose tolerance and hyperinsulinaemia of Zucker fatty rats were improved by the T-174 treatment in a dose dependent manner. Similar alleviation of glucose intolerance was also demonstrated in KK-A^y mice (data not shown).

Effects of the treatment of T-174 on insulin actions in vivo *and* ex vivo

Responsiveness to an exogenous insulin load was examined in insulin resistant KK-A^y mice after fasting for 20 h. In untreated KK-A^y mice, exogenous insulin up to 0.5 u kg^{-1} did not significantly decrease the blood glucose levels. In contrast, the hypoglycaemic effect of insulin was significantly augmented by treatment with T-174 (8.2 mg kg^{-1} day^{-1}, for 7 days) (Figure 4).

A hyperinsulinaemic euglycaemic clamp study was carried out with 28 μ kg^{-1} min^{-1} insulin infusion in Zucker fatty rats after 6 days of administration of T-174 (1.4 and 4.5 mg kg^{-1} day^{-1}). Steady state plasma insulin values were greater than 1000 μu ml^{-1} in all groups and there was no significant difference among the groups. The GIR_{60-120} for untreated Zucker fatty rats was only 24% of that for lean littermates. This severe insulin resistance was dose-dependently ameliorated in T-174-treated Zucker fatty rats (Figure 5).

To confirm the improvement of insulin action in peripheral tissues, we determined the metabolic changes in adipocytes from T-174-treated KK-A^y mice (16 mg kg^{-1} day^{-1}, for 7 days). There was a marked increase in both basal and insulin-stimulated glucose oxidation from 0.2 mM glucose in adipocytes from T-174-treated mice (Figure 6a). Although basal rates of lipogenesis from glucose were similar in adipocytes between untreated and T-174-treated mice, there was a significant increase in the insulin-stimulated lipogenesis in cells from drug treated animals at all insulin concentrations (Figure 6b).

We also measured the insulin binding in the same preparation of adipocytes. The total insulin binding to adipocytes from KK-A^y mice was increased by treatment with T-174 (Figure 7a). In a Scatchard analysis of the binding displacement curves, there was a parallel shift of the plots in T-174-treated adipocytes (Figure 7b). Thus the increase in insulin binding was due to an increase in the receptor number rather than a change in the affinity. Using a negative cooperative model for binding of insulin with its receptor (De Meyts & Roth, 1975), we calculated the total insulin receptor number to be 2.2×10^5 receptors $cell^{-1}$ and 5.4×10^5 receptors $cell^{-1}$ in adipocytes of untreated and T-174-treated mice, respectively. The cell size was not significantly changed by the T-174

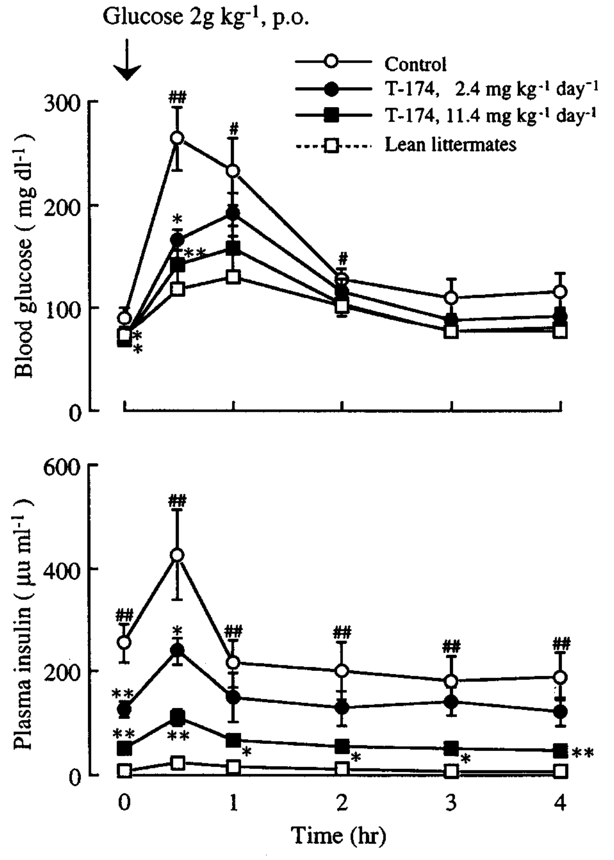

Figure 3 Effects of T-174 on blood glucose and plasma insulin levels in glucose loaded Zucker fatty rats. Ten-week-old male Zucker fatty rats were given T-174 (2.4 and 11.4 mg kg^{-1} day^{-1}) for 7 days. Control Zucker fatty rats, T-174-treated Zucker fatty rats, and their lean littermates were subjected to an oral glucose load (2 g kg^{-1}, at 0 h) on day 8 after a 20 h fast. Each symbol represents the mean and vertical lines show s.e.mean of six animals. #$P<0.05$, ##$P<0.01$ compared with lean littermates. *$P<0.05$ and **$P<0.01$ compared with control Zucker fatty rats.

Table 2 Effects of 6-day administration of T-174 on body weight, food intake, blood glucose, plasma insulin, and plasma lipid levels in Zucker fatty rats

Animal groups	*Body weight* (g)	*Food intake* (g day^{-1})	*Blood glucose* (mg dl^{-1})	*Plasma insulin* (μu ml^{-1})	*Plasma TG* (mg dl^{-1})	*Plasma NEFA* (μEq l^{-1})
Zucker fatty Control	580±13##	35	115±7##	659±75##	924±97##	434±16
Zucker fatty T-174 (1.4 mg kg^{-1} day^{-1})	568±16	39	105±4	341±57**	340±35**	225±8**
Zucker fatty T-174 (4.5 mg kg^{-1} day^{-1})	590±24	43	93±3*	152±14**	226±16**	223±40**
Lean littermates	331±8	23	89±2	27±4	160±19	429±44

T-174 was given to 12-week-old male Zucker fatty rats, weighing 445–595 g (average: 523 g), for 6 days. Each value represents as the mean±s.e.mean of six animals. ##$P<0.01$ compared with lean littermates. *$P<0.05$ and **$P<0.01$ compared with control. Abbreviations: TG, triglycerides; NEFA, non-esterified fatty acids.

treatment (untreated, 123±4 μm *vs* T-174-treated, 127±4 μm in diameter, $n=50$ cells).

Influence of T-174 on plasma magnesium levels

Plasma magnesium concentrations were lower in KK-A^y mice than normal ddY mice (Figure 8a). T-174 treatment increased plasma magnesium concentrations in KK-A^y mice in a dose-dependent manner and completely ameliorated the hypomagnesaemia at 16 mg kg^{-1} day^{-1}. There was a significant inverse relationship between plasma magnesium concentrations and blood glucose levels in these mice ($P<0.01$) (Figure 8b).

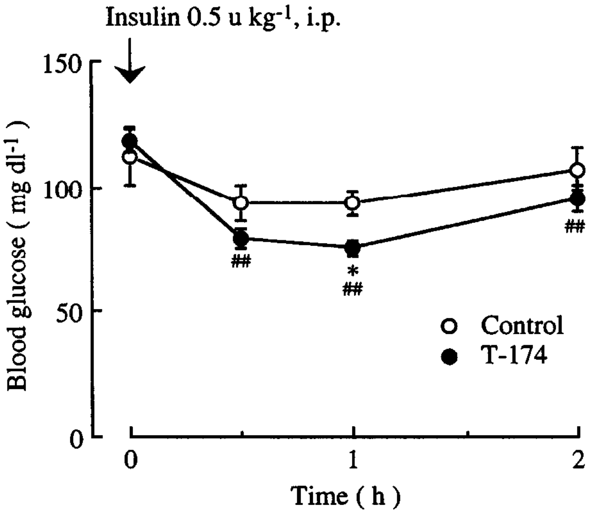

Figure 4 Effect of T-174 on blood glucose response to insulin in KK-A^y mice. Twelve-week-old male KK-A^y mice were given T-174 (8.2 mg kg^{-1} day^{-1}) for 7 days. Mice were given an i.p. injection of insulin (0.5 u kg^{-1}, at 0 h) on day 8 after a 20 h fast. Each symbol represents the mean and vertical lines show s.e.mean of six animals. $^*P<0.05$ compared with control. $^{\#\#}P<0.01$ compared with the initial blood glucose level.

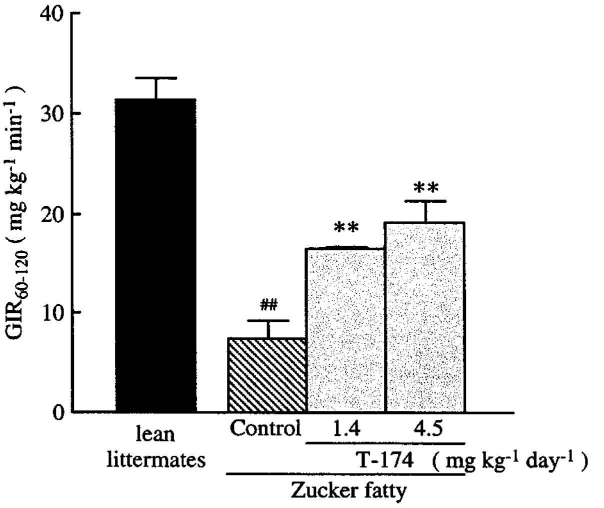

Figure 5 Effect of T-174 on glucose infusion rate during a hyperinsulinaemic euglycaemic clamp study in Zucker fatty rats. Twelve-week-old male Zucker fatty rats were given T-174 (1.4 and 4.5 mg kg^{-1} day^{-1}) for 6 days. Control Zucker fatty rats, T-174-treated Zucker fatty rats, and their lean littermates were subjected to a hyperinsulinaemic euglycaemic clamp after an overnight fast. Insulin was infused at 28 μ kg^{-1} min^{-1}. Following 1 h for stabilization, the glucose infusion rate (GIR$_{60-120}$) to maintain fasting plasma glucose level was measured. Each bar represents the mean and vertical lines show s.e.mean of four to five animals. $^{\#\#}P<0.01$ compared with lean littermates. $^{**}P<0.01$ compared with control Zucker fatty rats.

Effect of T-174 on glucose disposal and 2-deoxy-glucose uptake in cultured L6 myotubes

Cultured L6 myotubes were incubated with increasing concentrations of T-174 for 48 h. As shown in Figure 9a, T-174 increased the glucose consumption in a concentration-dependent manner. The EC$_{50}$ value of T-174 was 6 μM. This *in vitro* effect of T-174 was also more potent (about 6 times) than pioglitazone (EC$_{50}$; 37 μM). Similar results were found for [^{3}H]-2-deoxy-glucose uptake experiments; the EC$_{50}$ value of T-174 and pioglitazone were 4 and 25 μM, respectively (Figure 9b).

To examine the interaction between the actions of T-174 and insulin, L6 myotubes were incubated together with the two agents. The combined treatment of submaximal concentration of T-174 (3 μM) and insulin (1 μg ml^{-1}) resulted in an additive increase of glucose disposal (Table 3). Thus, T-174 enhanced both basal and insulin-stimulated glucose disposal in L6 myotubes.

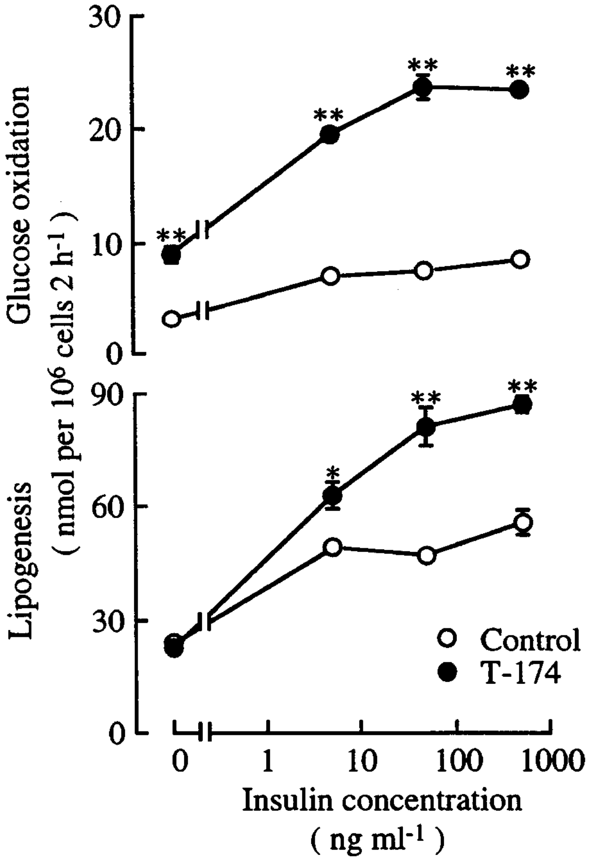

Figure 6 Glucose oxidation and lipogenesis from glucose by adipocytes isolated from KK-A^y mice after 7-day administration of T-174. T-174 (16 mg kg^{-1} day^{-1}) was administered to male KK-A^y mice (6-weeks-old) for 7 days. The adipocytes were incubated in the presence of 0.2 mM glucose with or without insulin at 37°C for 120 min. Each symbol represents the mean and vertical lines show s.e.mean of three observations. $^*P<0.05$ and $^{**}P<0.01$ compared with control.

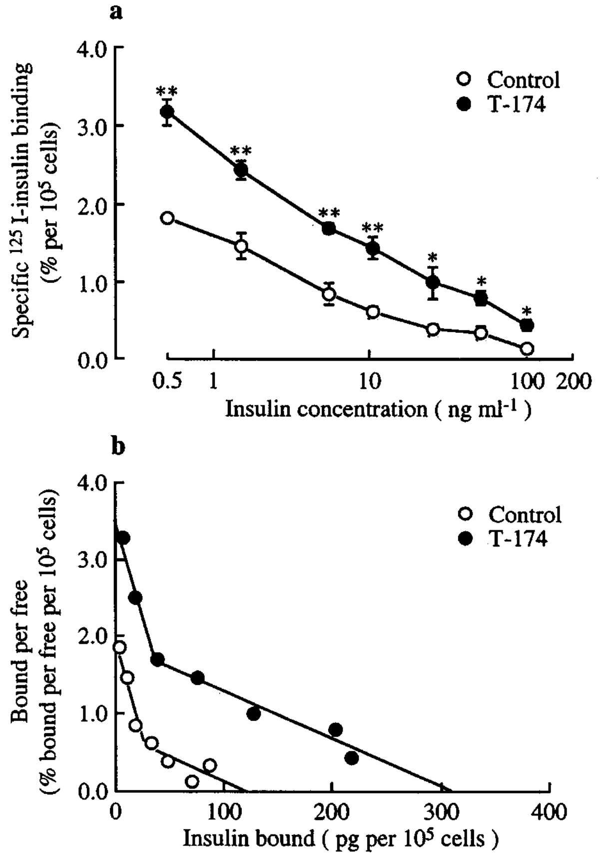

Figure 7 ^{125}I-insulin binding of adipocytes isolated from KK-A^y mice after 7-day administration of T-174. T-174 (16 mg kg^{-1} day^{-1}) was administered to male KK-A^y mice (6-weeks-old) for 7 days. The adipocytes were incubated with ^{125}I-insulin and various concentrations of unlabeled insulin at 24°C for 60 min. (a) Insulin binding displacement curves. (b) Scatchard plots. Each symbol represents the mean and vertical lines show s.e.mean of three observations. $^{*}P<0.05$ and $^{**}P<0.01$ compared with control.

Discussion

Oral administration of T-174 produced both a dose-dependent suppression of hyperglycaemia and an amelioration of glucose intolerance in insulin-resistant obese KK-A^y mice and Zucker fatty rats. The antihyperglycaemic effects of T-174 have been demonstrated in other insulin-resistant animal models including ob/ob, db/db, goldthioglucose-induced obese mice and dexamethasone-induced diabetic rats, but not in normal and streptozotocin-induced diabetic animals (unpublished observations). Thus the antihyperglycaemic profile of T-174 appears to depend on the presence of insulin similar to other antidiabetic TZDs (Fujiwara *et al.*, 1988; Ikeda *et al.*, 1990). In terms of ED$_{50}$ values for antihyperglycaemic effects, T-174 is more potent than ciglitazone, pioglitazone, troglitazone, and englitazone (Arakawa *et al.*, 1997) and is almost equipotent to BRL 49653 (Young *et al.*, 1995).

Since T-174 is one of the most potent antihyperglycaemic agents, we further characterized the metabolic effect of T-174 *in vivo* and *in vitro*. Improvement of glycaemic control by T-174 with a concomitant reduction in hyperinsulinaemia and insulin response to a glucose load suggests that the insulin resistance of peripheral tissues is positively influenced by T-174. An increase in insulin-mediated action measured in adipocytes from T-174-treated KK-A^y mice also supports this

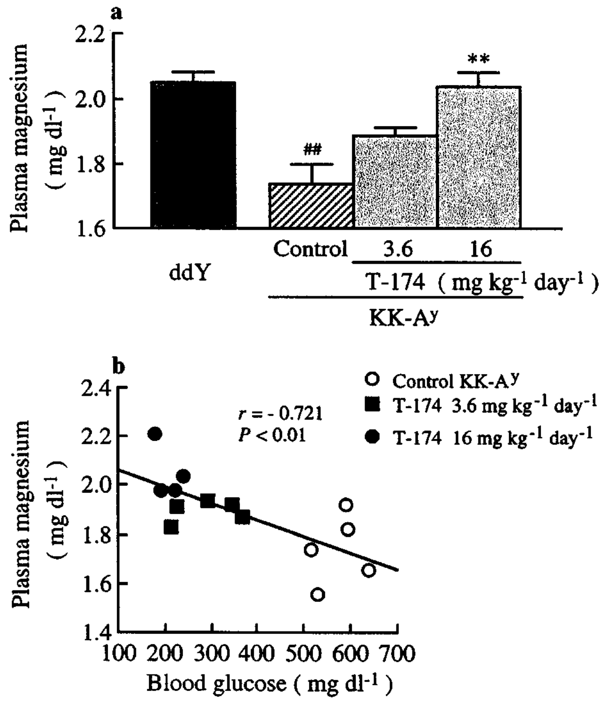

Figure 8 (a) Effect of T-174 on plasma magnesium concentration in KK-A^y mice. 15-week-old male KK-A^y mice were given T-174 (3.6 and 16 mg kg^{-1} day^{-1}) for 7 days. Each bar represents the mean and vertical lines show s.e.mean of five animals. $^{\#\#}P<0.01$ compared with normal ddY mice. $^{**}P<0.01$ compared with control. (b) Relationship between blood glucose and plasma magnesium levels in control and T-174-treated KK-A^y mice.

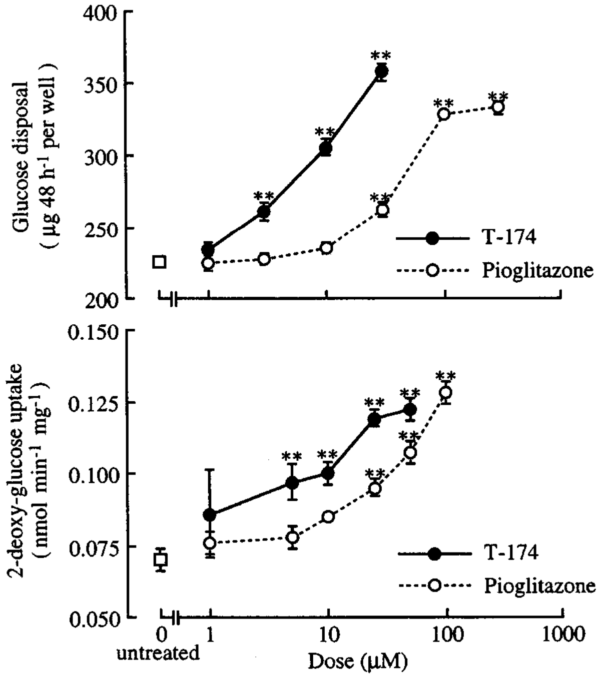

Figure 9 Effects of T-174 and pioglitazone on glucose disposal (a) and 2-deoxy-glucose uptake (b) in L6 myotubes. Cells were incubated with T-174 or pioglitazone for 48 h. Each symbol represents the mean and vertical lines show s.e.mean of four (glucose disposal) and three (2-deoxy-glucose uptake) independent experiments. $^{**}P<0.01$ compared with untreated.

Table 3 Additivity of the effects of T-174 and insulin on glucose disposal in L6 myotubes

T-174 (μM)	*Insulin* (μg ml^{-1})	*Glucose disposal* (μg 48 h^{-1} per well)
–	–	181.8±2.4
3	–	212.7±6.0*
–	1	251.2±11.6**
3	1	302.2±2.9**##

Cells were incubated with or without T-174 in the absence or the presence of insulin for 48 h and glucose consumption was determined. Each value represents as the mean±s.e.mean ($n=4$). Statistical analysis was done using Tukey-Krammer's multiple comparison test. *$P<0.05$ and **$P<0.01$ compared with basal state. ##$P<0.01$ compared with insulin alone.

conclusion. Furthermore, both the insulin tolerance test in KK-A^y mice and the hyperinsulinaemic euglycaemic clamp study in Zucker fatty rats clearly demonstrated that T-174 markedly enhanced overall insulin action *in vivo*. These results suggest that the primary action of T-174 is an improvement of peripheral insulin action.

The number of insulin binding sites of adipocytes was increased by 2.5 fold with T-174 treatment in KK-A^y mice. In contrast, there was no difference in the affinity of insulin receptors. An increased number of insulin binding sites without a change in affinity has also been reported to occur in adipocytes of Zucker fatty rats and ob/ob mice treated with troglitazone (Fujiwara *et al*., 1988) and BRL 49653 (Young *et al*., 1995), respectively. Thus up-regulation of insulin receptors may be a specific drug effect, that contributes to the enhanced insulin action. However, we cannot rule out the possibility that the increase in the number of insulin receptors is induced secondarily to the sustained reduction of plasma insulin levels, because hyperinsulinaemia induces down-regulation of insulin receptors (Koranyi *et al*., 1992).

In addition to the increase of insulin receptor numbers, augmentation of insulin-stimulated glucose metabolism including glucose oxidation and lipogenesis from glucose was also demonstrated in adipocytes from T-174-treated KK-A^y mice. The concentration response curves for insulin were displaced upward rather than shifted leftward by the T-174 treatment. According to the consideration of Kahn (Kahn, 1978), the upward shift would not result as a consequence of increased receptor number. Pioglitazone has been shown to facilitate the tyrosine phosphorylated insulin receptor and insulin receptor substrate 1 (IRS-1) levels and insulin-stimulated phosphatidylinositol (PI) 3-kinase in muscles of Wistar fatty rats (Hayakawa *et al*., 1996). In Chinese hamster ovary cells transfected with the human insulin receptor, pioglitazone has been reported to enhance the insulin-stimulated PI3-kinase activity without changing tyrosine phosphorylation of insulin receptors and IRS-1 (Zhang *et al*., 1994). Thus, alteration distal to the insulin receptor may also play an important role for the insulin enhancing action of T-174.

Pioglitazone (El-Kebbi *et al*., 1994) and troglitazone (Ciaraldi *et al*., 1995) have been shown to increase the transport of glucose in cultured myocytes, concomitant with the increase of glucose transporter proteins GLUT1 and GLUT4. In the present study, T-174 directly increased the glucose disposal as well as the 2-deoxy-glucose transport in L6 myotubes at concentrations as low as 3 μM. It appears, therefore, that enhanced glucose transport contributes to the augmented glucose disposal by T-174. In the preliminary study, we found that 5 μM was the maximal plasma concentration produced by 5 mg kg^{-1} p.o. of T-174 in KK-A^y mice. Thus, while conclusions drawn from *in vitro* studies should be cautiously extrapolated to *in vivo*, it is likely that a direct effect on the skeletal muscle also plays an important role for the hypoglycaemic action of T-174.

T-174 also decreased plasma levels of TG and NEFA like those of glucose and insulin. Since hyperinsulinaemia is tightly coupled with hypertriglyceridaemia (Reaven & Greenfield, 1981), reduction in insulin levels may be responsible for the decrease in plasma TG levels. The plasma NEFA levels seem to be lowered by T-174 through enhancing the antilipolytic action of insulin. In turn, reduction of NEFA levels improves glucose utilization via the glucose-fatty acid cycle (Randle *et al*., 1963) and relieves insulin resistance. Hypolipidaemic effects of T-174 thus could indirectly enhance the overall insulin action.

Magnesium supplementation can improve the impaired insulin sensitivity in both diabetic animal models (Balon *et al*., 1994, 1995) and patients with NIDDM (Paolisso *et al*., 1989). In the present study, the suppressed circulating magnesium levels in KK-A^y mice were markedly improved by T-174 treatment. Furthermore, there was an inverse correlation between plasma magnesium levels and blood glucose levels in these animals. Similarly, pioglitazone has been demonstrated to increase plasma ionized magnesium concentration in fructose-fed insulin resistant rats (Buchanan *et al*., 1995). Although the mechanism to increase the magnesium concentration remains to be determined, amelioration of decreased magnesium availability may contribute, at least in part, to alleviation of insulin resistance by T-174.

In summary, T-174 acts as a potent antihyperglycaemic, hypoinsulinaemic, and hypolipidaemic agent in insulin-resistant animal models by ameliorating the impaired insulin action. In addition to the effect on adipose tissues, improvement of insulin sensitivity by T-174 may involve multiple mechanisms including the normalization of plasma magnesium concentrations and the stimulation of glucose disposal in muscles.

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