Jikeikai Med J 2003 ; 50 : 99-107

Effect of Pioglitazone on Body Fat and Intramuscular Fat Accumulation in Otsuka Long- Evans Tokushima Fatty Rats : Its Relation to the Expression of Tumor Necrosis Factor-α mRNA

Yutaka MORI[1], Hideaki KOMIYA[2], Naoyuki KUROKAWA[3], and Naoko TAJIMA[4]

[1]*Department of Internal Medicine, National Higashi-Utsunomiya Hospital*
[2]*Exercise Physiology, Faculty of Education, Utsunomiya University*
[3]*Environmental Health Sciences, Tohoku University Graduate School of Medicine*
[4]*Division of Diabetes and Endocrinology, Department of Internal Medicine, The Jikei University School of Medicine*

ABSTRACT

The effects of pioglitazone on body fat and intramuscular fat accumulation and its relation to the expression of tumor necrosis factor (TNF)-α mRNA were examined in Otsuka Long-Evans Tokushima Fatty rats. Eight-week-old rats were divided into pioglitazone, glibenclamide, glibenclamide+pioglitazone, and control groups and monitored for 12 weeks. Body weight, adipose tissue weight, and triglyceride content in skeletal muscle were significantly higher in the pioglitazone group than in the control group and in the glibenclamide+pioglitazone group than in the glibenclamide group. Plasma levels of insulin, free fatty acids, triglycerides, and total cholesterol, TNF-α expression in adipose tissue, and triglyceride content in liver were significantly lower and in adipose tissue numbers of large adipocytes were lower and numbers of small adipocytes were greater in the pioglitazone group than in the control group and in the glibenclamide+pioglitazone group than in the glibenclamide group. These results suggest that pioglitazone decreases TNF-α expression in adipose tissue and decreases insulin resistance by reducing the size of adipocytes. In addition, pioglitazone might also affect adipocytes in skeletal muscle the same way as those in adipose tissue. (Jikeikai Med J 2003 ; 50 : 99-107)

Key words : pioglitazone, insulin resistance, TNF-α, body fat, intramuscular fat, Otsuka Long-Evans Tokushima Fatty rat

INTRODUCTION

The suggested mechanisms of action of thiazolidinedione derivatives at the organ level include the promotion of glucose uptake by the skeletal muscle system and inhibition of excessive gluconeogenesis in the liver[1]. Until recently, however, how thiazolidinedione derivatives lower insulin resistance at the molecular level was poorly understood. In 1994 *in vitro* studies showed that thiazolidinedione derivatives promote the differentiation of adipocytes[2-5]. These studies also showed that thiazolidinedione derivatives are synthetic ligands that activate peroxisome proliferation-activated receptor γ, a determining factor in adipocyte differentiation[2-5].

Received for publication, March 10, 2003
森　豊, 小宮　秀明, 黒川　修行, 田嶼　尚子
Mailing address : Yutaka MORI, Department of Internal Medicine, National Higashi-Utsunomiya Hospital, 2160 Shimookamoto, Kawachi-machi, Kawachi-gun, Tochigi 329-1193, Japan.
E-mail : moriyutakajp@yahoo.co.jp

More recently, troglitazone, a thiazolidinedione derivative, has been found to decrease insulin resistance by reducing the size of adipocytes in rats with hereditary obesity[6]. We have found that troglitazone conspicuously promotes accumulation of subcutaneous adipose tissue in mildly obese patients with type 2 diabetes[7]. Thereafter, many clinical studies regarding thiazolidinedione derivatives and body fat distribution were done[8–11]. In the present study, we investigated the effects of thiazolidinedione derivatives and sulfonylurea agents on the expression of tumor necrosis factor (TNF)-α mRNA in the adipose tissue of spontaneously diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats with visceral adiposity in relation to changes in the cellularity of adipose tissue.

Materials and Methods

Male OLETF rats[12–14] were provided by the Tokushima Research Institute of the Otsuka Pharmaceutical Co. (Tokushima, Japan). Eight weeks after birth, the rats were randomly divided into a pioglitazone group, a glibenclamide group, a glibenclamide+pioglitazone group, and a control group of 8 rats each. They were monitored for 12 weeks. The rats in the experimental groups were fed standard rat chow (MB-3; Funabashi Farm, Funabashi, Japan) mixed with 0.02% pioglitazone or 0.1% glibenclamide or both. Plasma levels of glucose, immunoreactive insulin (IRI), immunoreactive leptin (IRL), and lipids (triglyceride, total cholesterol, and free fatty acid) were measured every 6 weeks under nonfasting conditions. At the end of the experiment (20-week-old), the rats were fasted for 16 hours, and 2 g/kg oral glucose tolerance tests were performed. Blood was drawn a total of 5 times from the orbital venous plexus: before glucose loading and 30 minutes, 60 minutes, 120 minutes, and 180 minutes after glucose loading. Plasma levels of glucose and lipids were measured with the enzyme method, and plasma levels of IRI and IRL were measured with the radioimmunoassay method.

The weight of subcutaneous adipose tissue, retroperitoneal adipose tissue, mesenteric adipose tissue, adipose tissue around the epididymis and liver were measured. The left lobe of the liver and the left paravertebral muscle of each rat were harvested for lipid extraction and derivatization. The liver and muscle tissue were homogenized in a mixture of chloroform and methanol (2 : 1 vol/vol), and the total lipid extracts were prepared according to the method of Folch et al.[15].

The TNF-α mRNA expression volume was measured in retroperitoneal adipose tissue and skeletal muscle with the reverse transcriptase (RT)-polymerase chain reaction (PCR). This method involved using the GTC-$CsCl_3$ method to extract total RNA and RT to synthesize cDNA from total RNA. With cDNA as the template, PCR was used to amplify β-actin and TNF-α. Plots were created with the Southern blot technique. β-actin and TNF-α probes were hybridized, data were calculated, and then assessed with TNF-α/β actin.

Retroperitoneal and subcutaneous adipose tissues were fixed in 20% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H & E) and impregnated with silver, and areas occupied by adipocytes were measured with a NIH image analyzer. For each sample, approximately 340 adipocytes were measured to calculate the average area of adipocytes and determine the relative frequency of large and small adipocytes.

For statistical analysis, all numerical figures are expressed as mean±SD. The statistical analysis was performed by one way analysis of variance (ANOVA), followed by Scheffe's method as a post hoc test for any significant differences among groups, and the χ^2 test was used to assess the significance of differences in frequency between large and small adipocytes. Differences were considered significant when $p < 0.05$.

Results

Body weight and food intake were significantly higher in the pioglitazone group than in the control group and in the glibenclamide+pioglitazone group than in the glibenclamide group (Fig. 1).

Plasma levels of glucose, IRI, triglyceride, free fatty acid, and total cholesterol were significantly

lower in the pioglitazone group than in the control group (Fig. 2-a), whereas plasma levels of IRI and triglyceride in the glibenclamide group were significantly higher than in the control group (Fig. 2-b). Moreover, plasma levels of IRI, triglyceride, free fatty acid, and total cholesterol were significantly lower in the glibenclamide+pioglitazone group than in the glibenclamide group (Fig. 2-b).

On oral glucose tolerance testing (Fig. 3) plasma glucose levels before and 30, 60, and 120 minutes after glucose loading were significantly lower in the pioglitazone group than in the control group. Levels of IRI were significantly lower in the pioglitazone group than in the control group before glucose loading, but did not differ significantly thereafter. Neither plasma glucose levels nor IRI levels differed significantly between the glibenclamide group and the glibenclamide+pioglitazone group.

At the end of the experiment, the weights of subcutaneous, retroperitoneal, mesenteric, and epididymal adipose tissue and triglyceride content in skeletal muscle were significantly higher in the pioglitazone group than in the control group and in glibenclamide+pioglitazone group than in the glibenclamide group (Table 1). Liver weight and triglyceride content in liver were significantly lower in the pioglitazone group than in the control group and in the glibenclamide+pioglitazone group than in the glibenclamide group (Table 1).

The level of TNF-α mRNA expression in adipose tissue was significantly lower in the pioglitazone group than in the control group and significantly higher in the glibenclamide group than in the control group, However, TNF-α mRNA expression was significantly lower in the glibenclamide+pioglitazone group than in the glibenclamide group (Table 1). TNF-α mRNA expression in the skeletal muscle did not differ significantly between the groups (Table 1).

Histopathologic examination showed that small adipocytes in retroperitoneal adipose tissue were particularly abundant in the pioglitazone group (Fig. 4-a) and in the glibenclamide+pioglitazone group

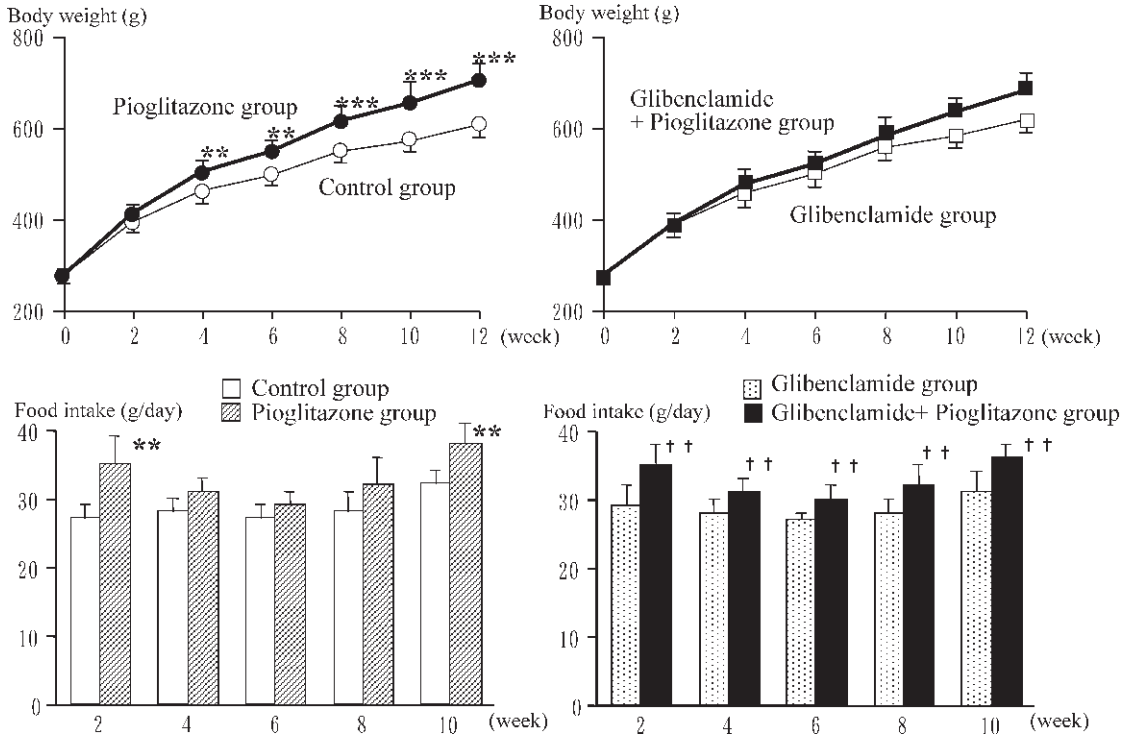

Fig. 1. Changes in body weights and food intakes in pioglitazone group (solid circle, striped column) and control group (open circle, open column) (Left) and glibenclamide+pioglitazone group (solid square, solid column) and glibenclamide group (open square, stippled column) (Right). $^{**}p<0.01$, $^{***}p<0.001$, vs value of control group, $^{\dagger\dagger}p<0.01$, vs value of glibenclamide group.

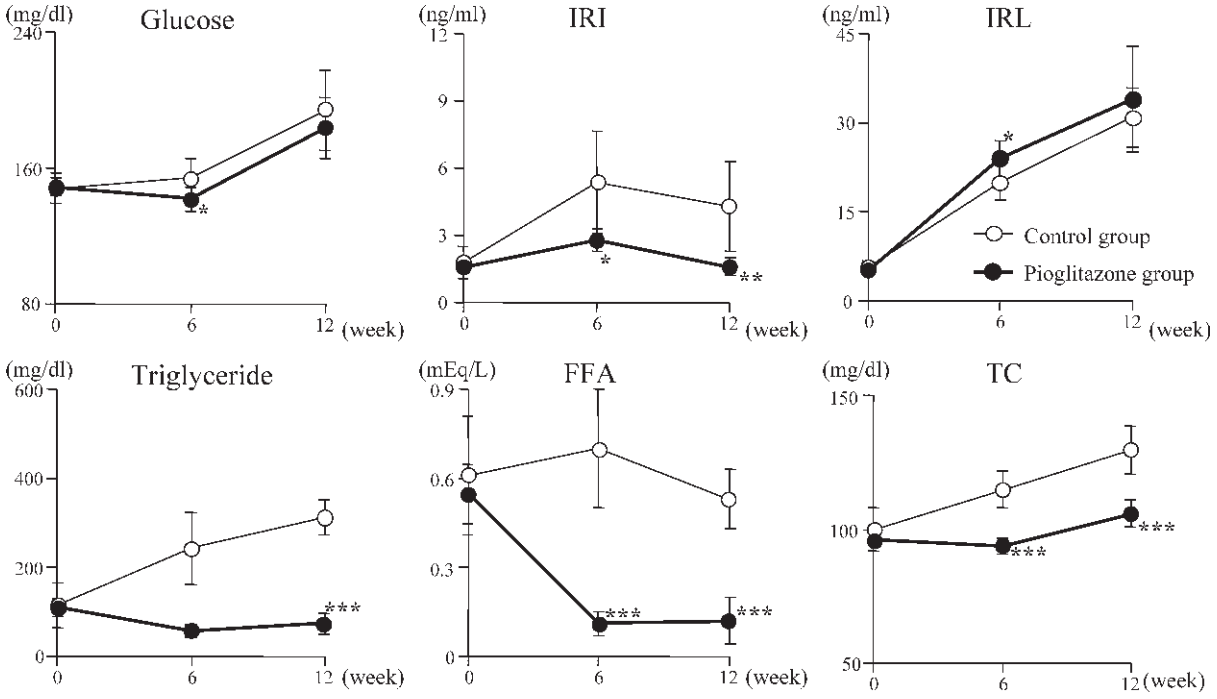

Fig. 2-a

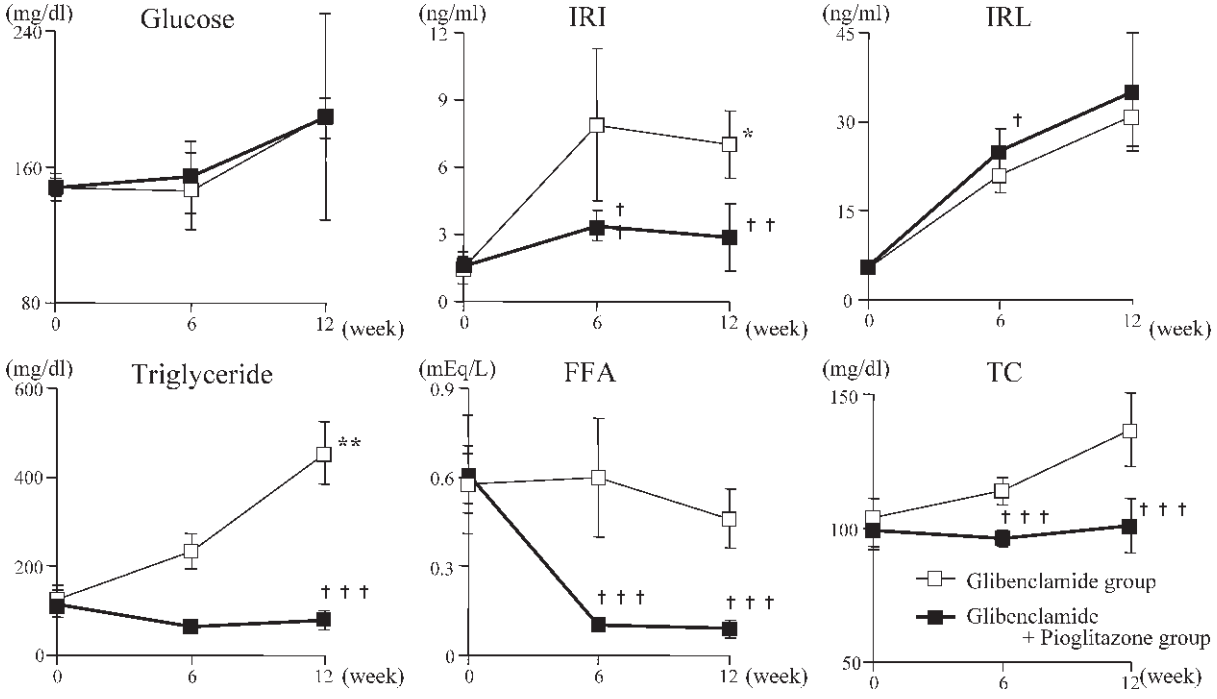

Fig. 2-b

Fig. 2. Changes in plasma glucose, insulin, leptin, triglyceride, free fatty acid and total cholesterol levels in pioglitazone group (solid circle) and control group (open circle) (a), and glibenclamide+pioglitazone group (solid square) and glibenclamide group (open square) (b). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, vs value of control group, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$, vs value of glibenclamide group.

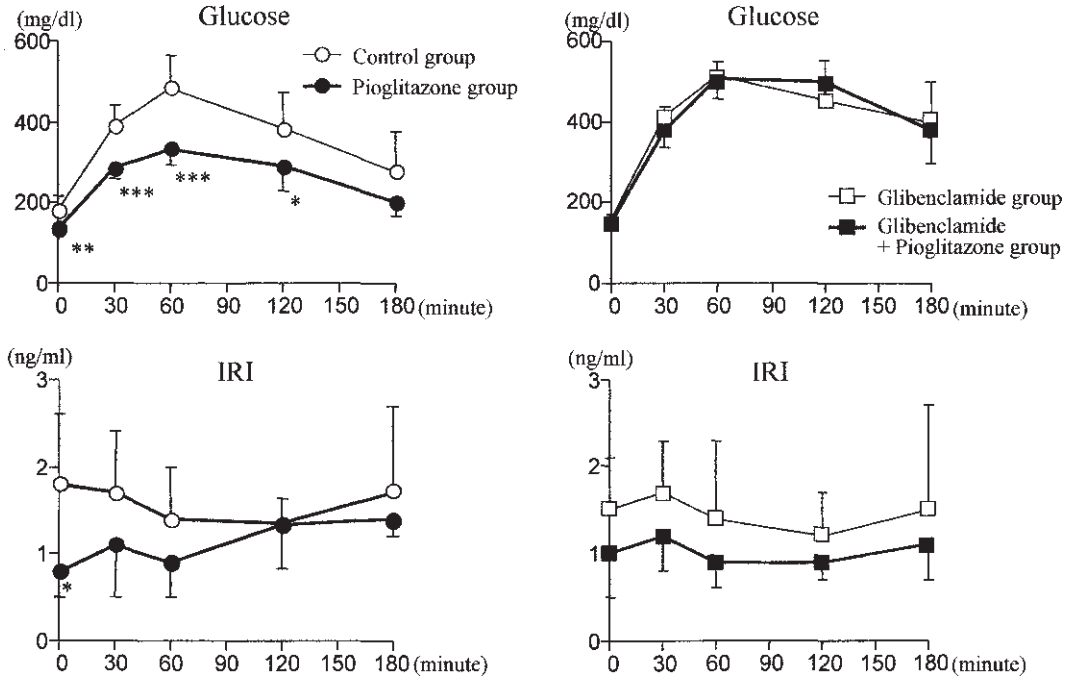

Fig. 3. Plasma glucose levels and insulin response after 2 g/kg oral glucose loading in pioglitazone group (solid circle) and control group (open circle), and glibenclamide+pioglitazone group (solid square) and glibenclamide group (open square). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs value of control group.

Table 1. Adipose tissue weight, liver weight, triglyceride content in skeletal muscle and liver, and levels of TNF-α expression in adipose tissue and skeletal muscle in the control group, pioglitazone group, glibenclamide group, and glibenclamide+pioglitazone group.

Variable	Control	Pioglitazone	Glibenclamide	Glibenclamide+ Pioglitazone
Adipose tissue weight (g)				
Subcutaneous	9.48±0.48	14.53±1.87***	8.95±0.92	14.0±1.67†††
Retroperitoneal	12.64±0.94	17.46±2.26***	12.35±1.18	16.8±1.89†††
Mesenteric	9.48±0.57	12.04±1.46***	9.81±0.34	11.3±1.10††
Epididymal	6.85±0.85	10.55±1.54***	6.83±0.84	9.46±0.66†††
Liver weight (g)	19.35±1.14	15.78±1.37***	20.43±1.62	16.91±0.84†††
Triglyceride content (mg/g wet tissue)				
Skeletal muscle	26.6±10.5	53.1±22.4*	27.2±11.1	50.3±14.5††
Liver	62.0±8.4	28.4±10.8***	54.8±9.4	30.2±6.1†††
TNF-α mRNA expression (TNF-α/β actin)				
Adipose tissue	0.91±0.44	0.33±0.09**	2.19±1.08*	0.64±0.24††
Skeletal muscle	0.47±0.75	0.20±0.35	0.96±1.11	0.15±0.42

*$p<0.05$, **$p<0.01$, ***$p<0.001$, vs value of control group, ††$p<0.01$, †††$p<0.001$, vs value of glibenclamide group

(Fig. 4-b). The average area of adipocytes was significantly ($p<0.001$) smaller in the pioglitazone group than in the control group, significantly ($p<0.001$) larger in the glibenclamide group than in the control group, and significantly ($p<0.001$) smaller in the glibenclamide+pioglitazone group than in the glibenclamide group (Table 2).

Frequency distribution tables showed that adipocyte area in the pioglitazone group was shifted downward compared with that it in the control group.

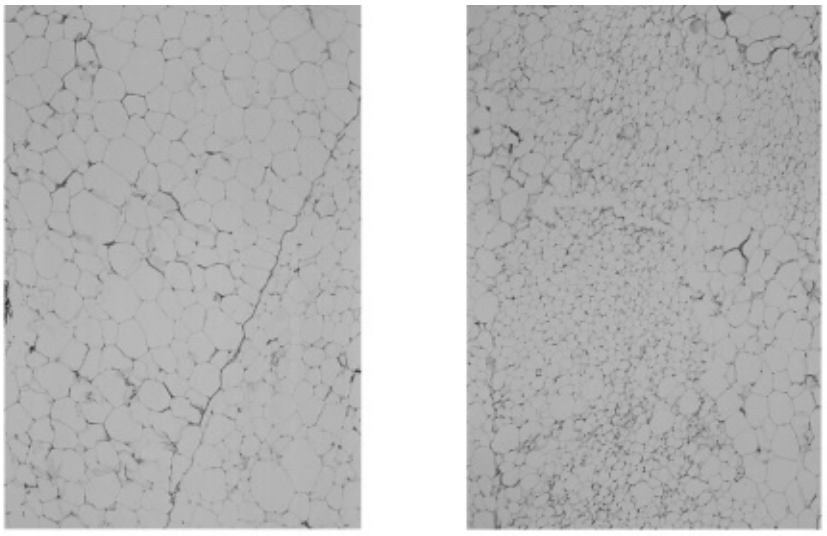

Fig. 4-a

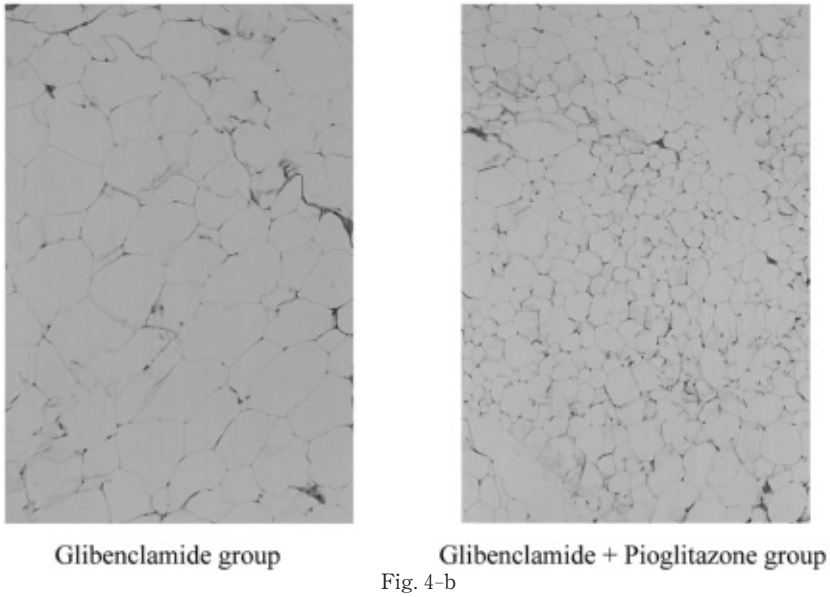

Fig. 4-b

Fig. 4. The histological views of adipose tissue in pioglitazone group and control group (a) (H & E, ×15), and in glibenclamide+pioglitazone group and glibenclamide group (b) (H & E, ×25).

Table 2. Average size of adipocytes in the control, pioglitazone, glibenclamide, and glibenclamide+pioglitazone groups.

Study group	Number	Average area (μm^2)	SD	P value
Control	338	10,755.3	6,192.6	
Pioglitazone	340	7,972.9	3,630.7	<0.001 vs control
Glibenclamide	338	13,764.6	7,036.1	<0.001 vs control
Glibenclamide+ Pioglitazone	339	8,108.9	3,756.0	<0.001 vs glibenclamide

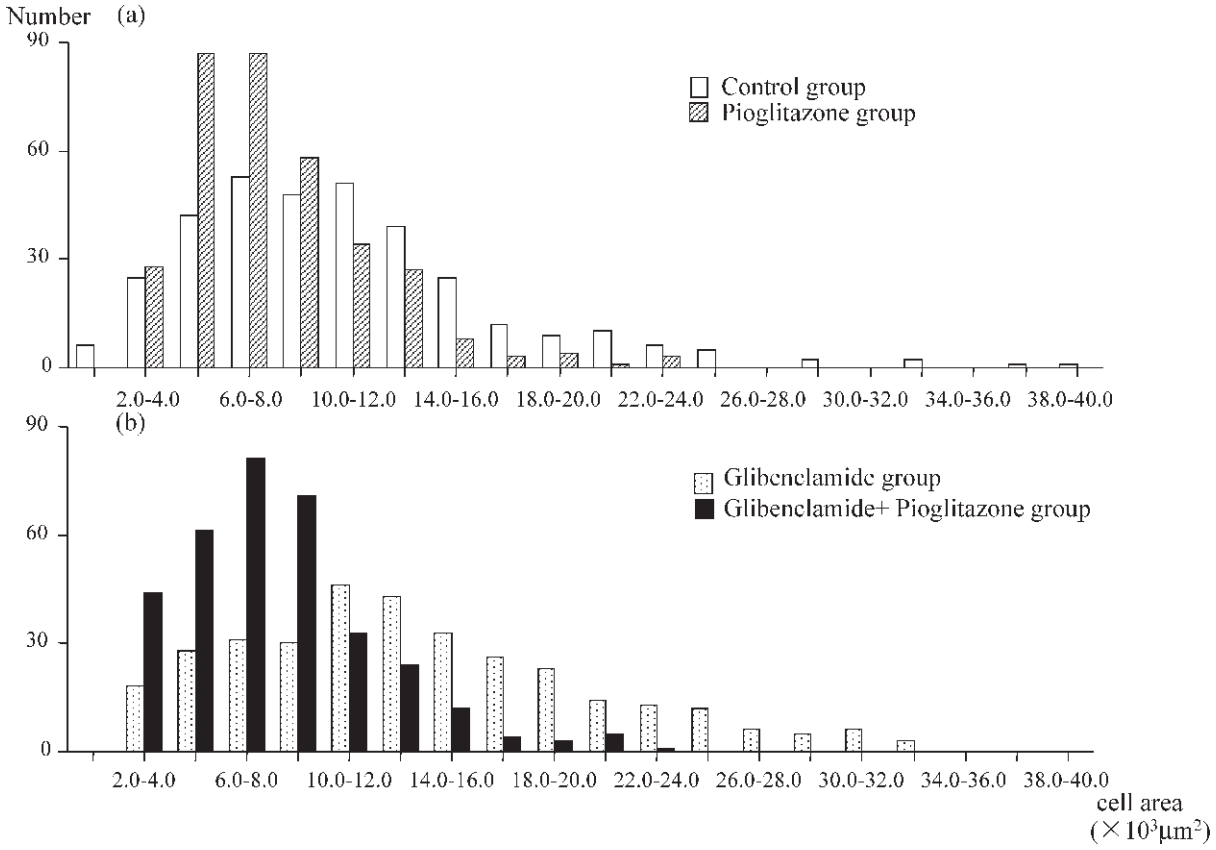

Fig. 5. Distribution of adipocyte in adipose tissue in pioglitazone group (striped column) and control group (open column) (a), and glibenclamide+pioglitazone group (solid column) and glibenclamide group (stippled column) (b).

Table 3. Frequency (numbers) of small and large adipocytes in the control, pioglitazone, glibenclamide, and glibenclamide+pioglitazone groups.

Study group	Less than 25th percentile (small adipocytes)	More than 75th percentile (large adipocytes)	*P* value
Control	85	84	
Pioglitazone	137	27	<0.001 vs control
Glibenclamide	52	157	<0.001 vs control
Glibenclamide+Pioglitazone	119	27	<0.001 vs glibenclamide

Adipocyte area peaked between 6,000 and 12,000 μm^2 in the control group and between 4,000 and 8,000 μm^2 in the pioglitazone group. In the glibenclamide group, however, adipocyte size had generally shifted upward compared with that in the control group. Furthermore, the adipocyte area was generally less in the glibenclamide+pioglitazone group than in the glibenclamide group (Fig. 5).

The number of adipocytes with areas below the 25th percentile (6,397.8 μm^2) and above the 75th percentile (13,398.5 μm^2) of the control group differences were revealed at the 0.1% level between the control and pioglitazone groups; at the 0.1% level between the control and the glibenclamide group; and at the 0.1% level between the glibenclamide and the glibenclamide+pioglitazone groups (Table 3). Adipocyte size was generally smaller in subcutaneous tissue than in retroperitoneal adipose tissue, while the trend was

similar to those seen in the retroperitoneal adipose tissue.

DISCUSSION

In our experiment, expression of TNF-α mRNA in the retroperitoneal adipose tissue was significantly higher in the glibenclamide group than in the control group but was significantly lower in the glibenclamide+pioglitazone group than in the glibenclamide group. Furthermore, the glibenclamide group showed a significantly higher number of large adipocytes in the retroperitoneal adipose tissue than did the control group, whereas the number of small adipocytes was significantly higher and the number of large adipocytes was significantly lower in the glibenclamide+pioglitazone group than in the glibenclamide group. These findings strongly suggest a close relation between TNF-α mRNA expression and cellularity in adipose tissue, although the mechanism of this relation is unclear.

We found that the number of large adipocytes was less and the number of small adipocytes was greater in the pioglitazone group than in the control group. In Zucker fatty rats, troglitazone was reported to increase the number of small adipocytes and reduced the number of large adipocytes[6]. These findings agree with the results of our experiment with pioglitazone.

However, glibenclamide is thought to boost expression of TNF-α mRNA in adipose tissue by inducing adipocyte hypertrophy. The OLETF rats used in this experiment are an animal model of diabetes[8–10] in which insulin secretion in response to glucose loading is gradually delayed with age. In persons with such delayed insulin secretion, glibenclamide might stimulate insulin secretion for prolonged periods of time and thus induce adipocyte hypertrophy, especially in visceral adipose tissue.

Adams et al.[16] have performed *in vitro* studies of the effects of rosiglitazone, another thiazolidinedione derivative, on the differentiation of human preadipocytes from different adipose tissue sites. Although levels of peroxisome proliferation-activated receptor γ expression were similar in subcutaneous adipose tissue and visceral adipose tissue, rosiglitazone promoted the differentiation of preadipocytes to mature adipocytes only in subcutaneous adipose tissue. These findings agree with clinical results[7] indicating that troglitazone markedly promotes accumulation of subcutaneous adipose tissue in mildly obese patients with type 2 diabetes. However, these results differed from our results in rats in that pioglitazone increased the percentage of small adipocytes in retroperitoneal adipose tissue, rather than in subcutaneous adipose tissue, while suppressing TNF-α mRNA expression in adipose tissue. The reason for the difference in the adipose tissue sites influenced by thiazolidinedione derivatives between the patients with type 2 diabetes and OLETF rats is unclear. However, one study in Zucker fatty rats has shown that TNF-α mRNA expression is inhibited to a greater degree in retroperitoneal adipose tissue than in subcutaneous adipose tissue[6], a result that agrees with our findings in OLETF rats. The discrepancies between studies might be explained by such factors as the duration of treatment and differences between animal models and humans in insulin levels and in adipocyte sensitivity to different thiazolidinedione derivatives.

Our results suggest that thiazolidinedione derivatives, when administered as monotherapy, promote the differentiation of preadipocytes to small, mature adipocytes, which produce and secrete substances that increase insulin sensitivity. Where insulin secretion is stimulated by glibenclamide and adipocytes undergo hypertrophy, thiazolidinedione derivatives appear to promote differentiation of preadipocytes into small, mature adipocytes. In addition, these agents might reduce the size of large, mature adipocytes. Apoptosis or cell division might be involved in this reduction of large adipocytes. Indeed, some studies have suggested that apoptosis of adipocytes is involved[17] and, therefore, emphasize the need for research to investigate nuclear chromatin agglutination and apoptosis corpuscles.

Our results suggest that in patients with diabetes with visceral adiposity, sulfonylurea agents may enlarge adipocytes in visceral adipose tissue and increase TNF-α mRNA expression in adipose tissue,

leading to increased insulin resistance. Thiazolidinedione derivatives might increase the percentage of small adipocytes, reduce the number of large adipocytes, and, consequently, reduce the overexpression of TNF-α mRNA, thus decreasing insulin resistance. In addition, pioglitazone might also affect adipocytes in skeletal muscle.

References

1. Suter SL, Nolan JJ, Wallace P, Gumbiner B, Olefsky JM. Metabolic effects of new oral hypoglycemic agent CS-045 in NIDDM subjects. Diabetes Care 1992 ; 15 : 193-203.
2. Lehmann JM, Moore LB, Smith-Oliver T.A, Wilkinson WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). J Biol Chem 1995 ; 270 : 12953-6.
3. Ohsumi J, Sakakibara S, Yamaguchi J, Miyadai K, Yoshioka S, Fujiwara T, et al. Troglitazone prevents the inhibitory effect of inflammatory cytokines on insulin-induced adipocyte differentiation in 3T3-L1 cells. Endocrinology 1994 ; 135 : 2279-82.
4. Szalkowski D, White-Carrington S, Berger J, Zhang J. Bolodeoku. Antidiabetic thiazolidinediones block the inhibitory effect of tumor necrosis a on differentiation, insulin-stimulated glucose uptake, and gene expression in 3T3-L1 cells. Endocrinology 1995 ; 136 : 1474-81.
5. Lambe KG, Tugwood JD. A human peroxisome-proliferator-activated receptor γ is activated by inducers of adipogenesis, including thiazolidinedione drugs. Eur J Biochem 1996 ; 239 : 1-7.
6. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, et al. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. J Clin Invest 1998 ; 101 : 1354-61.
7. Mori Y, Murakawa Y, Okada K, Horikoshi H, Yokoyama J, Yajima N, et al. Effect of troglitazone on body fat distribution in type 2 diabetic patients. Diabetes Care 1999 ; 22 : 908-12.
8. Katoh S, Hata S, Matsushima M, Ikemoto S, Inoue Y, Yokoyama J, et al. Troglitazone prevents the rise in visceral adiposity and improves fatty liver associated with sulfonylurea therapy : a randomized controlled trial. Metabolism 2001 ; 50 : 414-7.
9. Nakamura T, Funahashi T, Yamashita S, Nishida M, Nishida Y, Takahashi M, et al. Thiazolidinedione derivative improves fat distribution and multiple risk factors in subjects with visceral fat accumulation-double-blind placebo-controlled trial. Diabetes Res Clin Pract 2001 ; 54 : 181-90.
10. Miyazaki Y, Mahankali A, Matsuda M, Mahankali S, Hardies J, Cusi K, et al. Effect of pioglitazone on abdominal fat distribution and insulin sensitivity in type 2 diabetic patients. J Clin Endocrinol Metab 2002 ; 87 : 2784-91.
11. Hirose H, Kawai T, Yamamoto Y, Taniyama M, Tomita M, Matsubara K, Okazaki Y, Ishii T, Oguma Y, Takei I, Saruta T : Effects of pioglitazone on metabolic parameters, body fat distribution, and serum adiponection levels in Japanese male patients with type 2 diabetes. Metabolism 2002 ; 51 : 314-7.
12. Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. Spontaneous long-term hyperglycemic rat with diabetic complications, Otsuka Long-Evans Tokushima fatty (OLETF) Strain. Diabetes 1992 ; 41 : 1422-8.
13. Mori Y, Yokoyama J, Nemoto M, Katoh S, Nishimura M, Ikeda Y. Characterization of a new genetically obese-hyperglycemic OLETF rat. Jikeikai Med J 1992 ; 39 : 349-59.
14. Mori Y, Ikeda Y. Similarity and dissimilarity between the OLETF rats and obese subjects with NIDDM, In : Shima K editor. Obesity and NIDDM. Lessons from the OLETF rat. Amsterdam : Elsevier Science BV ; 1999. p. 237-44.
15. Folch L, Less M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957 ; 226 : 497-507.
16. Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L,et al. Activators of peroxisome proliferator-activated receptor-γ have depot-specific effects on human preadipocyte differentiation. J Clin Invest 1997 ; 100 : 3149-53.
17. Prins JB, Niesler CU, Winlerford CM, Bright NA, Siddle K, O'Rahilly S, et al. Tumor necrosis factor-α induces apoptosis of human adipose cells. Diabetes 1997 ; 46 : 1939-44.