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Effects of Pioglitazone vs Glibenclamide on Postprandial Increases in Glucose and Triglyceride Levels and on Oxidative Stress in Japanese Patients with Type 2 Diabetes

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To investigate the relationship between insulin resistance, postprandial hyperglycemia, postprandial hyperlipidemia, and oxidative stress in type 2 diabetes, changes in postprandial glucose, triglyceride, and nitrotyrosine levels vs baseline after diet loading were examined in type 2 diabetic patients given pioglitazone (PG) or glibenclamide (GB). Twenty-four outpatients with type 2 diabetes treated with oral PG for 6 mo (BMI, 26.3 ± 0.9; HbA1c, 8.2 ± 0.2%) and 10 type 2 diabetic patients treated with GB (BMI, 27.4 ± 1.6; HbA1c, 8.1 ± 0.2%) at our institutions were compared. These patients were given meal tolerance tests (MTT; each consisting of energy 400 kcal, protein 8.7 g, fat 22.4 g, carbohydrate 41 g) before and 6 mo after administration of either agent. PG produced a significant decrease in FPG, HbA1c, HOMA-R, and TG levels in the subjects compared to baseline. In contrast, GB significantly decreased FPG and HbA1c levels, while not affecting HOMA-R and TG values. While PG produced a significant increase in LPL, HDL-cholesterol, and adiponectin levels, GB did not affect these values. At MTT 6 mo after PG administration, insulin levels before and 4 h after MTT, free fatty acid (FFA) levels 1, 2, and 4 h after MTT, glucose, TG, and RLP-TG levels before and 1, 2, 4, and 6 h after MTT were significantly decreased compared to baseline. At MTT 6 mo after GB administration, while a significant decrease in fasting and 2 h, postprandial glucose values compared to baseline MTT levels was observed, fasting and postprandial TG and RLP-TG levels remained unchanged compared to baseline. After 6 mo of PG and GB administration, serum nitrotyrosine levels before and after MTT were significantly decreased compared to baseline in both groups, while the decrease in nitrotyrosine levels before and after MTT was more marked in the subjects given PG. Our study results suggest that PG suppresses increases in postprandial glucose and TG levels, and improves insulin resistance; and, in addition, that PG may have a favorable impact on oxidative stress in type 2 diabetic patients.

Key Words: Pioglitazone; glibenclamide; postprandial hyperglycemia; postprandial hyperlipidemia; oxidative stress; nitrotyrosine.

Introduction

A series of dramatic changes are known to occur postprandially even in healthy individuals, which involves an increase in blood glucose that lowers the ability to scavenge free radicals, an activation of the polyol metabolic pathway resulting in an increase in sorbitol, followed by an increased generation of reactive oxygen species (ROS), all of which combine to lead to increased oxidative stress. In recent years, oxidative stress is drawing attention as a crucial risk factor linking diabetes and atherosclerosis, where repeated, temporary rises in postprandial glucose levels result in endothelial damage, which is known to form part of the pathogenesis of atherosclerosis from its early stage. Additionally, an elevation of postprandial glucose and triglyceride levels has been associated with an increase in oxidative stress, thereby causing endothelial dysfunction *(1–3)*. Furthermore, insulin-resistant states and obesity have also been associated with increased oxidative stress *(4–6)*.

In order to elucidate the relationship between insulin resistance, postprandial hyperglycemia, postprandial hyperlipidemia, and oxidative stress, our current study investigated postprandial glucose, triglyceride, RLP-triglyceride levels after diet loading in patients given the oral insulin sensitizer pioglitazone (PG) vs those in patients given the oral insulin secretagogue glibenclamide (GB).

Results

There were no differences between the treatment groups with regard to baseline patient characteristics. BMI values

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Table 1
Changes in Laboratory Test Findings in Pioglitazone- and Glibenclamide-Treated Patients

	Pioglitazone group			Glibenclamide group		
	0M	3M	6M	0M	3M	6M
Number	24	24	24	10	10	10
BMI	26.3 ± 0.9	26.5 ± 1.0*	26.9 ± 1.0**†	27.4 ± 1.6	27.7 ± 1.5	27.7 ± 1.6*
FPG (mg/dL)	190 ± 10.8	145 ± 6.5**	143 ± 7.3**	172.9 ± 7.7	148 ± 8.4**	137 ± 11*
HbA1c (%)	8.2 ± 0.2	7.5 ± 0.2**	7.2 ± 0.2***	8.1 ± 0.2	7.3 ± 0.2*	7.1 ± 0.2**
Insulin (μU/mL)	10.7 ± 1.7	8.0 ± 1.2*	6.9 ± 1.1**††	12.3 ± 1.7	14.7 ± 2.6	15.1 ± 1.6*
HOMA-R	5.23 ± 1.2	2.60 ± 0.4*	2.24 ± 0.4**††	5.35 ± 0.9	5.56 ± 1.3	5.06 ± 0.8
LPL (ng/mL)	43.9 ± 2.7	48.8 ± 3.4**	55.1± 4.3**†	45.6 ± 2.6	47.5 ± 3.0	48.8 ± 2.6
TG (mg/dL)	179 ± 17	132 ± 11*	120 ± 11***	166 ± 13	176 ± 14	162 ± 16
HDL-C (mg/dL)	54.0 ± 2.7	61.4 ± 3.2***	63.3 ± 3.3***	54.2 ± 5.3	52.7 ± 6.0 5	2.5 ± 5.0
TC (mg/dL)	212 ± 6.5	211 ± 5.9	215 ± 5.9	215 ± 13.5	202 ± 11.6	206 ± 10.6
PAI-1 (ng/mL)	36.3 ± 4.5	—	28.1 ± 3.2	41.8 ± 4.9	—	43.9 ± 3.6
TNF-α (pg/mL)	20.5 ± 2.0	—	15.0 ± 2.2*	20.9 ± 6.4	—	23.4 ± 5.1
Adiponectin (μg/mL)	7.8 ± 0.67	—	18.4 ± 2.2***	7.94 ± 1.2	—	7.86 ± 1.0
hs-CRP (ng/mL)	2582 ± 587	—	1995 ± 599	2640 ± 848	—	2933 ± 1137

Mean ± SEM.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs 0M, †$p < 0.05$, ††$p < 0.01$ vs 3M.

after the start of treatment with PG and GB were shown to be significantly increased in both PG- and GB-treated patients compared to baseline. Fasting plasma glucose (FPG) levels, HbA1c, HOMA-R, TG, and TNF-α values were significantly decreased in PG-treated patients compared to baseline. In contrast, in GB-treated patients, FPG levels and HbA1c values were significantly reduced; however, HOMA-R values, TG and TNF-α levels remained unchanged (Table 1). Furthermore, while pre-heparin LPL mass, HDL-C and adiponectin levels were significantly increased in PG-treated patients, these variables remained unchanged in GB-treated patients (Table 1).

Insulin levels before and 4 h after MTT, FFA levels 1, 2, and 4 h after MTT as well as plasma glucose, TG, and RLP-TG levels before, 1, 2, 4, and 6 h after MTT, 6 mo after the start of PG treatment, were significantly decreased in PG-treated patients, compared to pretreatment levels before and after MTT (Figs. 1 and 2). In contrast, 6 mo after the start of GB treatment, fasting and 2 h-postprandial glucose levels were significantly decreased compared to baseline MTT levels, no marked changes were observed in insulin, FFA, TG, and RLP-TG levels in GB-treated patients (Figs. 1 and 2). The areas under the postprandial glucose, postprandial TG, and postprandial RLP-TG curves at MTT 6 mo after the start of treatment were significantly decreased in PG-treated patients compared to baseline, while no changes in these parameters were observed in GB-treated patients (Fig. 3).

While nitrotyrosine levels and the area under the nitrotyrosine curve before and after MTT 6 mo, after the start of treatment with both PG and GB, were shown to be significantly decreased in both PG- and GB-treated patients compared to baseline, these changes were more marked in PG-treated patients (Fig. 4).

Discussion

We observed a significant increase in BMI values in both treatment groups after the start of treatment, and while why and how this occurred remains unclear, we assumed that this could be attributed to the fact that both treatment groups included many obese patients, as shown by their baseline BMI values, who might have failed to adhere to the diet and exercise therapy prescribed, which likely led to the observed increase in BMI values.

Our study results showed that PG and GB resulted in a comparable decrease in HbA1c values, but led to disparate results with regard to HOMA-R values, TG levels, and TNF-α levels. Furthermore, while PG also led to an increase in pre-heparin LPL mass, HDL-cholesterol, and adiponectin levels, GB did not affect these parameters. Again, while PG led to a significant decrease in pre-MTT and post-MTT insulin, glucose, FFA, TG, RLP-TG levels, GB only showed a significant decrease in fasting and 2-h postprandial glucose levels but did not affect fasting and postprandial insulin, FFA, TG, or RLP-TG levels, with the decrease in nitrotyrosine levels with GB less marked than with PG. These findings suggest that postprandial glucose and TG elevations, insulin-resistant states, as well as hyperinsulinemia, combine to lead to an aggravation of oxidative stress *(1–6)*.

Oxidative stress is defined as an imbalance between free radical formation that causes damage and antioxidant activity that counteracts free radical formation in the body, where oxidative stress resulting from insufficient antioxidant con-

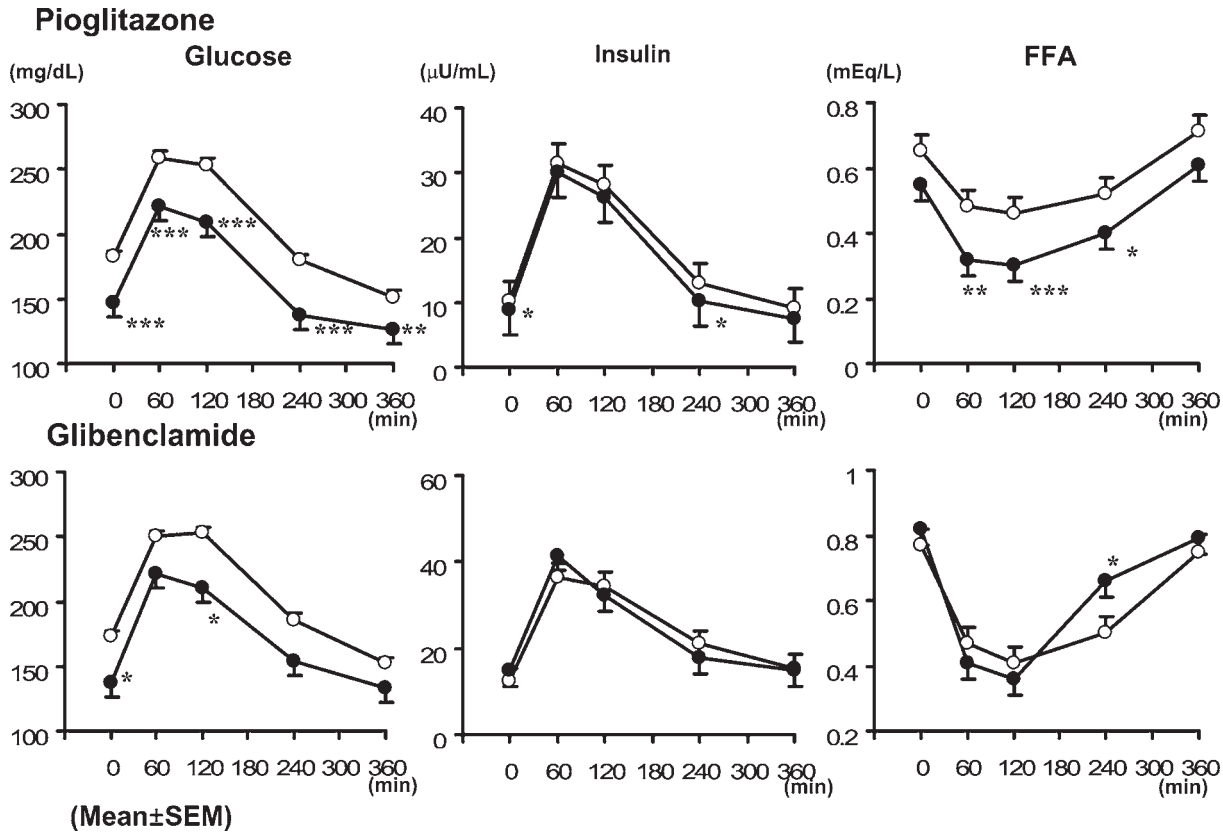

Fig. 1. Changes in blood glucose, insulin, and free fatty acid levels before and after MTT, before and 6 mo after the start of treatment with pioglitazone (PG) or glibenclamide (GB). Open circle, MTT before treatment; closed circle, MTT after 6 mo. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs MTT before treatment.

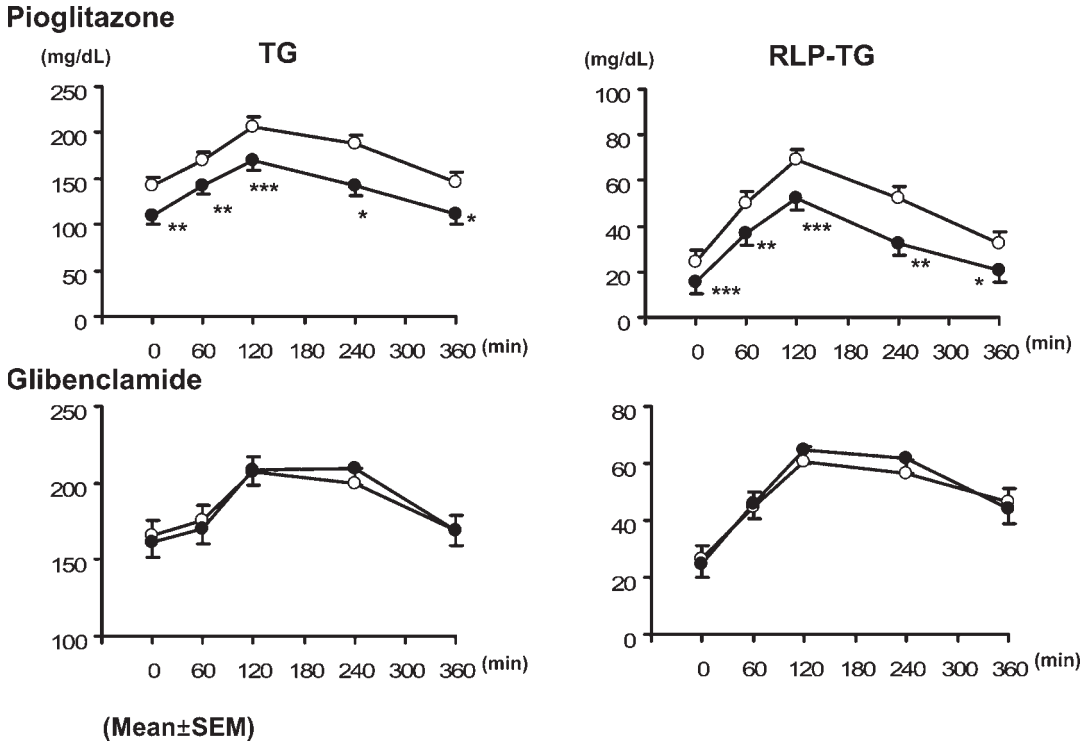

Fig. 2. Changes in TG and RLP-TG levels before and after MTT, before and 6 mo after the start of treatment with PG or GB. Open circle, MTT before treatment; closed circle, MTT after 6 mo. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs MTT before treatment.

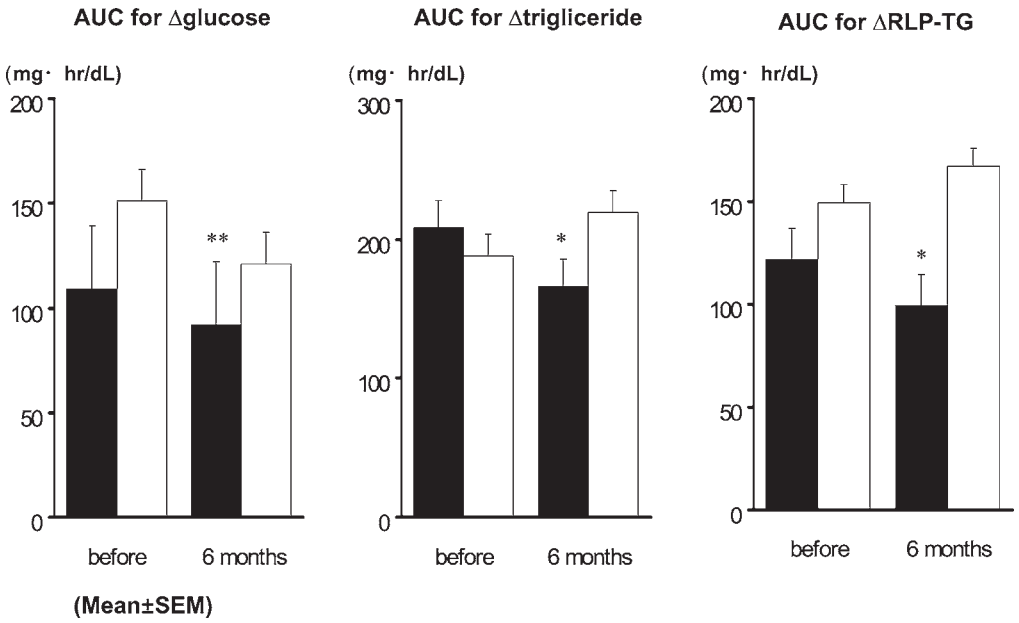

Fig. 3. Changes in the areas under the postprandial glucose, TG, and RLP-TG curve before and after MTT, before and 6 mo after the start of treatment with PG or GB. ■, PG; □, GB. *$p < 0.05$, **$p < 0.01$ vs MTT before treatment.

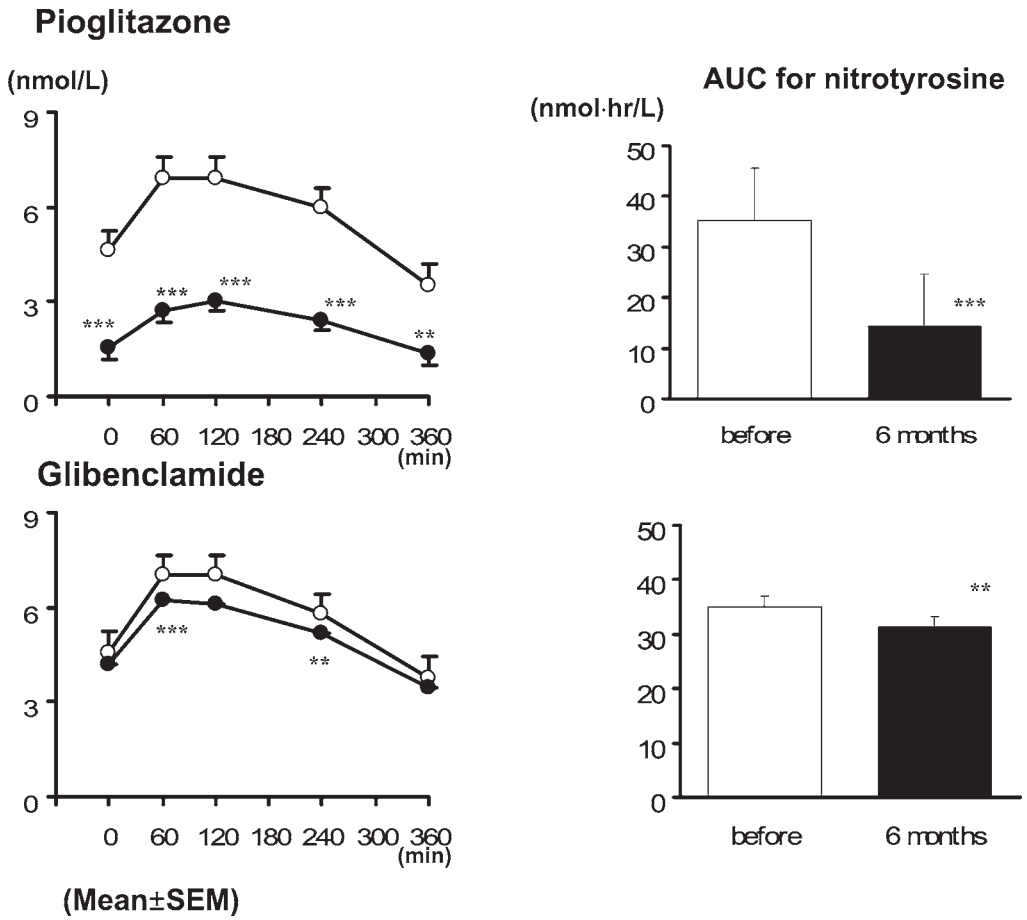

Fig. 4. Changes in nitrotyrosine levels and the area under the nitrotyrosine curve before and after MTT, before and 6 mo after the start of treatment with PG or GB. Open circle, MTT before treatment; closed circle, MTT after 6 mo. ■, PG; □, GB. **$p < 0.01$, ***$p < 0.001$ vs MTT before treatment.

sumption brings about a degeneration of proteins and lipids, consequently causing damage to the liver, kidney, lung, vascular wall, skin, and muscle tissues. Again, while nitric oxide (NO) is known as a key physiologically active substance that works to mediate some important functions such as vasodilation and secretion of neurotransmitters, NO is also known to interact with superoxide (O_2^-) to turn into cytotoxic peroxynitrite ($ONOO^-$, PN), thereby becoming implicated in the pathogenesis of various inflammatory diseases *(7–9)*. PN works to modify proteins by means of nitration through a series of reactions, and alters its functions. Of the changes thus brought about by PN, nitrotyrosine, a nitrated form of tyrosine residues, has drawn attention in recent years as being implicated in the pathogenesis of lifestyle-related diseases such as atherosclerosis *(10,11)*. Ceriello et al. subjected healthy volunteers to a hyperglycemic glucose clamp study where plasma glucose levels were acutely elevated to 15 mmol/L for 2 h, and found that blood nitrotyrosine concentrations in these subjects increased by twofold during the clamp study *(12,13)*. Again, studies in cultured endothelial cells demonstrated that intermittent hyperglycemic spikes are associated with elevated nitrotyrosine levels and increased apoptosis *(14)*.

We evaluated nitrotyrosine levels as a marker for oxidative stress, and found that the nitrotyrosine levels in our subjects were lower than those reported by Ceriello et al, while we used more or less the same ELISA method for determination of nitrotyrosine concentrations as in the study of Ceriello et al. *(13)*. One possible reason for this discrepancy could be that our study used a different primary antibody with a different sensitivity and specificity to the nitrotyrosine–BSA or patient serum samples, and this may consequently have resulted in lower nitrotyrosine values in our study. Another reason could be that our study results may have reflected differences in patient background, including race, severity of disease, factors that combined to constitute the sources of oxidative stress, and the ability to defend against oxidative stress. Therefore, given that the nitrotyrosine ELISA assay still remains to be standardized and various nitrotyrosine levels reported in the literature do not allow fair comparison, at the moment, comparison of nitotyrosine levels need to be made only between studies conducted under more or less identical conditions.

On another front, various reports demonstrated that fat accumulation is well correlated with oxidative stress or that overriding ROS production is observed in the adipose tissue of obese mice, suggesting that increased ROS in tissues characterized by fat accumulation is an early instigator of the metabolic syndrome in place *(6)*. Experiments in cultured adipocytes also showed that elevated FFA levels lead to an increase in NADPH oxidases, followed by an increase in ROS in the presence of decreased antioxidant enzyme activity, where ROS induces an abnormal production of adipocytokines, such as adiponectin, PAI-1, IL-6, and monocyte chemotactic protein-1, in local adipose tissues, as well as a systemic increase in oxidative stress *(6)*. Against this background, our study findings suggest that pioglitazone induced a qualitative change *(15)* in the adipose tissue of obese, type 2 diabetic patients, and ameliorated excess production of ROS by inducing a decrease in postprandial FFA levels, followed by an decrease in TNF-α levels and an increase in adiponectin levels as well as a decrease in nitrotyrosine levels.

To date, a number of studies have been reported that suggest pioglitazone possesses antioxidant properties *(16,17)*. Our study results further suggest that pioglitazone not only ameliorated postprandial glucose spikes and postprandial triglyceride elevations thereby improving insulin resistance, but also likely affected the adipocytes and had direct effects on oxidative stress. Additionally, while we did not evaluate any other markers for oxidative stress than nitrotyrosine or measure FFA levels in local adipose tissues, the significant postprandial decrease in FFA levels observed in the PG-treated patients appears to suggest that PG not only produced a qualitative change in the adipose tissue but also enhanced the action of insulin, thus leading to the enhanced inhibition of lipolysis in the adipose tissue. Again, it is suggested that this decrease in FFA levels was also associated with a corresponding decrease in FFA levels in local adipose tissues, thereby affecting the production of ROS or adipocytokines in the adipose tissue. Thus, pioglitazone appears to exert a variety of anti-atherosclerotic, rather than merely glucose-lowering, effects, and may be positioned as a unique agent possessing at once glucose-lowering and anti-atherosclerotic properties.

Patients and Methods

Patients

Twenty-four outpatients with type 2 diabetes [age, 65 ± 4; male/female, 15/9; body mass index (BMI), 26.3 ± 0.9; fasting plasma glucose (FPG), 190 ± 10.8 mg/dL; HbA1c, 8.2 ± 0.2%)] were treated with oral PG (15–30 mg/d) for 6 mo to compare the treatment outcome with that in 10 patients (age, 69 ± 4; male/female, 3/7; BMI, 27.4 ± 1.6; FPG, 172.9 ± 7.7 mg/dL; HbA1c, 8.1 ± 0.2%) treated with oral GB (1.25–2.5 g/mg/d). Although all patients had received nutritional counseling and had been encouraged to exercise, their glycemic status was poorly controlled with dietary treatment. They received PG monotherapy or GB monotherapy for 6 mo. Patients receiving oral fibrates, and those with a history of ischemic heart disease and/or stroke were excluded from the study. The present study was conducted with the approval of the ethics committee of Utsunomiya National Hospital, the National Hospital Organization, and all subjects gave informed consent to the study protocol.

Protocol

HbA1c, HOMA-R, lipid (total cholesterol, TC; triglyceride, TG; high density lipoprotein-cholesterol, HDL-C),

pre-heparin lipoprotein lipase (LPL) mass, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α), adiponectin, high-sensitivity C-reactive protein (hs-CRP) levels were measured at appropriate time points, and meal tolerance tests (MTT), each consisting of energy 400 kcal, protein 8.7 g, lipids 22.4 g, and glucose 41 g, were performed before and 6 mo after treatment. MTT involved blood sampling before, 1, 2, 4, and 6 h after meals to determine plasma glucose, insulin, TG, FFA, remnant-like particle-triglyceride (RLP-TG), and nitrotyrosine levels.

Plasma glucose levels were determined by the glucose dehydrogenase method. Hemoglobin A1c (HbA1c) was measured by cation exchange high-performance liquid chromatography (Bio-Rad Laboratories, Hercules, CA, USA). Serum lipids (TG, FFA, TC, HDL-C) were measured enzymatically using enzyme reagents (L-Type TG H, Wako Pure Chemicals, Osaka, Japan; NEFA-SS, Eiken Chemical, Tokyo, Japan; L-Type CHO H, Wako Pure Chemicals, Osaka, Japan; Cholestest N HDL, Daiichi Pure Chemicals, Tokyo, Japan). Pre-heparin LPL mass was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibody against bovine milk LPL, as described by Kobayashi et al. *(18)*. For the assay, a kit from Daiichi Pure Chemicals (Tokyo, Japan) was used. Insulin and adiponectin levels were determined using commercial enzyme immunoassay kits (LS Eiken Insulin Kit, Eiken Chemical, Tokyo, Japan; and an adiponectin ELISA kit, Otsuka, Tokushima, Japan). PAI-1 was determined with enzyme-linked immunosorbent assay (EIA) (Monozyme Inc, Hϕrsholm, Denmark), and TNF-α, with high sensitivity EIA (R&D Systems Inc, Minneapolis, MN, USA). High-sensitivity C-reactive protein (hs-CRP) was measured by latex nephelometry assay (N High Sensitivity CRP, Dade Behring, Marburg GmbH, Marburg, Germany).

Serum nitrotyrosine levels were determined by the direct ELISA method with all samples measured in duplicate, where patient serum samples and control/standard nitrotyrosine-BSA (Cayman Chemical Company, MI, USA) were diluted to 1/3 with carbonate buffer, pH 9.6, and were adsorbed on to plates (4°C overnight). On the next day, nonspecific binding was blocked by BSA 0.1% in PBS-T (PBS plus 0.05% of Tween 20), and monoclonal anti-nitrotyrosine antibody (Cayman Chemical Company), diluted to 1/10000 (final concentration), was added to the samples, and the samples were incubated at 37°C. The plates were washed and incubated with peroxidase-labeled anti-mouse IgG (Vector Laboratories, Ltd., Peterborough, UK) at 37°C for 1 h. The plates were washed again, and the peroxidase reaction product was generated using tetramethylbenzidine (TMB) microwell peroxidase substrate, and the reaction was stopped by adding H_2SO_4. The optical density was measured at 410 nm, and the nitrotyrosine concentration in each sample was calculated based on the optical density concurrently obtained for the standard nitrotyrosine BSA.

Statistical Analysis

For statistical analysis, all numerical values were represented as mean ± SEM. The paired *t*-test was used to test for significance ($p < 0.05$). Baseline differences between the treatment groups were tested for significance using an unpaired *t*-test.

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