Introduction

Obesity-linked insulin resistance is a key factor in the development of type 2 diabetes and metabolic syndrome, eventually leading to micro- and macrovascular complications. Thiazolidinediones (TZDs) have been shown to enhance the actions of insulin in the liver and skeletal muscle in animal models of obesity-linked insulin resistance and also in subjects with type 2 diabetes [1, 2]. TZDs bind to and activate the ligands of peroxisome proliferator-activated receptor-γ (PPAR-γ), thereby promoting adipose tissue differentiation, an increase in the number of small adipocytes, and an increase in the expression and secretion of adiponectin [3]. Increased production of adiponectin has been reported to improve insulin sensitivity, including glucose metabolism in the liver, where adiponectin decreases gluconeogenesis via activation of AMP-activated protein kinase (AMPK) [4, 5]. These beneficial effects of TZDs have made them attractive agents for the treatment of type 2 diabetes mellitus [6]. There has been growing recognition, however, that treatment with TZDs is associated with body weight gain and edema. The body weight gain and edema associated with the use of TZDs can be a cause for concern, as it may be a harbinger or sign of congestive heart failure (CHF), especially in subjects with type 2 diabetes who are at increased risk for cardiovascular diseases (CVD). These side effects of TZDs have limited their clinical use [7]. Recently, it has been reported that the body weight gain associated with low-dose TZD treatment may be significantly more modest than that associated with high-dose TZD treatment [8, 9]. On the other hand, low-dose TZDs may not produce adequate improvement in insulin resistance and glucose intolerance [10, 11].

Beraprost sodium (BPS), which is a stable prostaglandin I_2 (PGI_2) analog, binds to the PGI_2 receptor and increases cellular cyclic adenosine monophosphate (cAMP) levels in the platelets and smooth muscle cells [12, 13] and has been used clinically for the treatment of peripheral arterial disease and primary pulmonary hypertension [14, 15]. In addition, BPS increases the expression levels of eNOS mRNA and protein through cAMP-, protein kinase A (PKA)-, and cAMP-responsive element-mediated pathways in endothelial cells [16]. We recently reported that restoration of insulin-induced eNOS activation by BPS treatment improved the skeletal muscle glucose uptake in the HF diet-fed obese mice [17].

In the present study, we investigated whether combined treatment with low-dose pioglitazone (PIO-L) plus BPS might yield sufficient improvement of the insulin resistance and glucose intolerance in obese diabetic KKAy mice and also whether the use of this combination treatment might prevent PIO-induced body weight gain. The results revealed that combined PIO-L plus BPS treatment improved hyperglycemia and insulin resistance to a degree similar to that obtained with high-dose PIO (PIO-H) treatment; in addition, no significant change in the body weight of the animals was noted. These results suggest that combined use of PIO-L plus BPS may be one of the useful therapeutic strategies for patient with type 2 diabetes.

Materials and methods

Animal preparation and experimental protocol

Male KKAy mice (5–7 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and housed individually under a 12-h light/dark cycle. The animals were allowed access to water ad libitum. Pioglitazone was suspended in 0.5 % methylcellulose (MC). Low-dose pioglitazone (PIO-L; 3 mg/kg/day), high-dose pioglitazone (PIO-H; 30 mg/kg/day), or vehicle (0.5 % MC) was administered orally once daily for 20 consecutive days. The total administration volume was 0.01 ml/g body weight of the mice. On day 6 after the start of PIO treatment, a mini-osmotic pump (model 1002, ALZA Corp.) filled with BPS (1 mg/kg/day) or saline was implanted subcutaneously in the KKAy mice for 14 days (Supplementary Figure S1). The animals' body weight was monitored daily. The rate of increase in body weight was calculated by dividing the difference in body weight between the first and final day by that on the first day. BPS and pioglitazone were provided by Toray Industries, Inc., Tokyo, Japan. The animal care and experimental procedures used in this study were approved by the Animal Care Committee of the University of Tokyo.

Hyperinsulinemic-euglycemic clamp

The hyperinsulinemic-euglycemic clamp study was carried out as described previously [18], with slight modifications. In brief, an infusion catheter was inserted into the right jugular vein of the test animals under sodium pentobarbital anesthesia. The clamp studies were performed under conscious and unstressed conditions after the animals had been denied access to food for 4 h. To measure the glucose infusion rate (GIR), a primed continuous infusion of insulin (Humulin R, Lilly) was administered (10 mU/kg/min), and the blood glucose concentration, monitored every 5 min, was maintained at approximately 120 mg/dl by administration of glucose [5 g of glucose per 10 ml enriched to about 20 % with [6,6-$^2H_2$]glucose (Sigma)] for 120 min. Blood samples (20 μl) were obtained via tail-tip bleeds at 90, 105, and 120 min for determination of the rate of glucose disappearance (Rd). Rd was calculated according to nonsteady-state equations, and hepatic glucose production (HGP) was calculated as the difference between the Rd and GIR.

Glucose tolerance test

After being denied access to food for 16 h, the mice were loaded orally with glucose at 1.5 mg/g body weight, as described previously [18]. Blood samples were collected at 15, 30, 60, and 120 min after glucose loading. Blood glucose measurements were performed using an automatic glucometer (Glutest Ace, Sanwa Chemical Co., Nagoya, Japan).

Blood sample assay

Plasma insulin and adiponectin levels were determined using the mouse insulin (Morinaga, Yokohama, Japan) and adiponectin (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) ELISA kits, respectively. Plasma triglyceride (TG), free fatty acid (FFA), and total cholesterol (T-chol) (Wako Pure Chemical Industries Ltd., Osaka, Japan) were assayed by enzymatic methods.

Statistical analysis

Values are expressed as mean ± SEM. The statistical significance of all possible pairwise differences of the means was determined by the Tukey-Kramer test. Statistical significance was set at $p \leq 0.05$.

Results

Combined PIO-L plus BPS treatment did not produce any significant increase in body weight of KKAy mice

Although a significant increase in body weight was observed in KKAy mice treated with PIO-H, as compared with that in the vehicle-treated mice, from 14 days after the start of treatment, no such increase was observed in the animals treated with BPS, PIO-L, or PIO-L plus BPS (Fig. 1a). The rate of body weight increase was significantly higher in the animal group treated with PIO-H than in the groups treated with vehicle, BPS, or PIO-L plus BPS (Fig. 1b). The rate of body weight increase in the animals treated with BPS, PIO-L, or PIO-L plus BPS was not significantly different from that in the animals treated with vehicle (Fig. 1b).

Combined PIO-L plus BPS treatment tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance in KKAy mice

Although the fasting blood glucose levels were not significantly different among the treatment groups (Fig. 2a), fasting plasma insulin levels in KKAy mice treated with

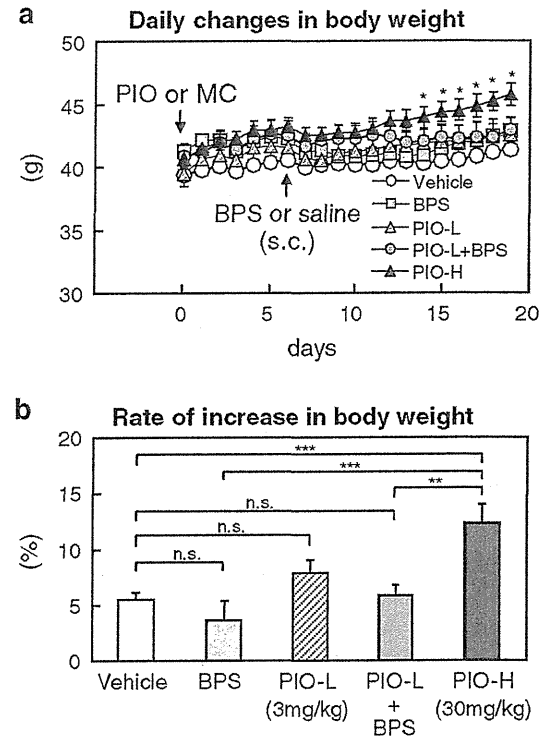

Fig. 1 Combined PIO-L plus BPS treatment produced no significant increase in body weight in KKAy mice. Daily changes **(a)** and rate of increase of the body weight **(b)** in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Values are mean ± SEM ($n = 5$–12). $*p \leq 0.05$ **(a)**, $**p \leq 0.01$, $***p \leq 0.001$ **(b)**. *n.s.* not significant

BPS, PIO-L, PIO-L plus BPS, or PIO-H were significantly decreased as compared with the values in the vehicle-treated KKAy mice (Fig. 2b). These data suggest that both BPS and PIO treatment improved the insulin resistance in KKAy mice. In fact, in the hyperinsulinemic-euglycemic clamp studies carried out in these mice, the glucose infusion rate (GIR) in KKAy mice treated with BPS, PIO-L plus BPS, or PIO-H were significantly increased compared with the rate in the vehicle-treated KKAy mice (Fig. 2c). Consistent with results in respect to the fasting insulin levels, the GIR tended to be higher in the animals receiving PIO-L treatment than in those treated with vehicle. Although no increase in the glucose disappearance rate (Rd) was observed in either the animals treated with PIO-L or those treated with PIO-H, the Rd was significantly higher in the animals treated with BPS or PIO-L plus BPS than in those treated with vehicle (Fig. 2d). In contrast, hepatic glucose production (HGP) was significantly suppressed following PIO-H treatment and tended to be lower in the animals treated with PIO-L or PIO-L plus BPS than in those treated with vehicle. No such decrease in HGP was observed following BPS treatment. These data suggest that PIO and BPS improved the hepatic and skeletal muscle insulin resistance, respectively (Fig. 2d, e).

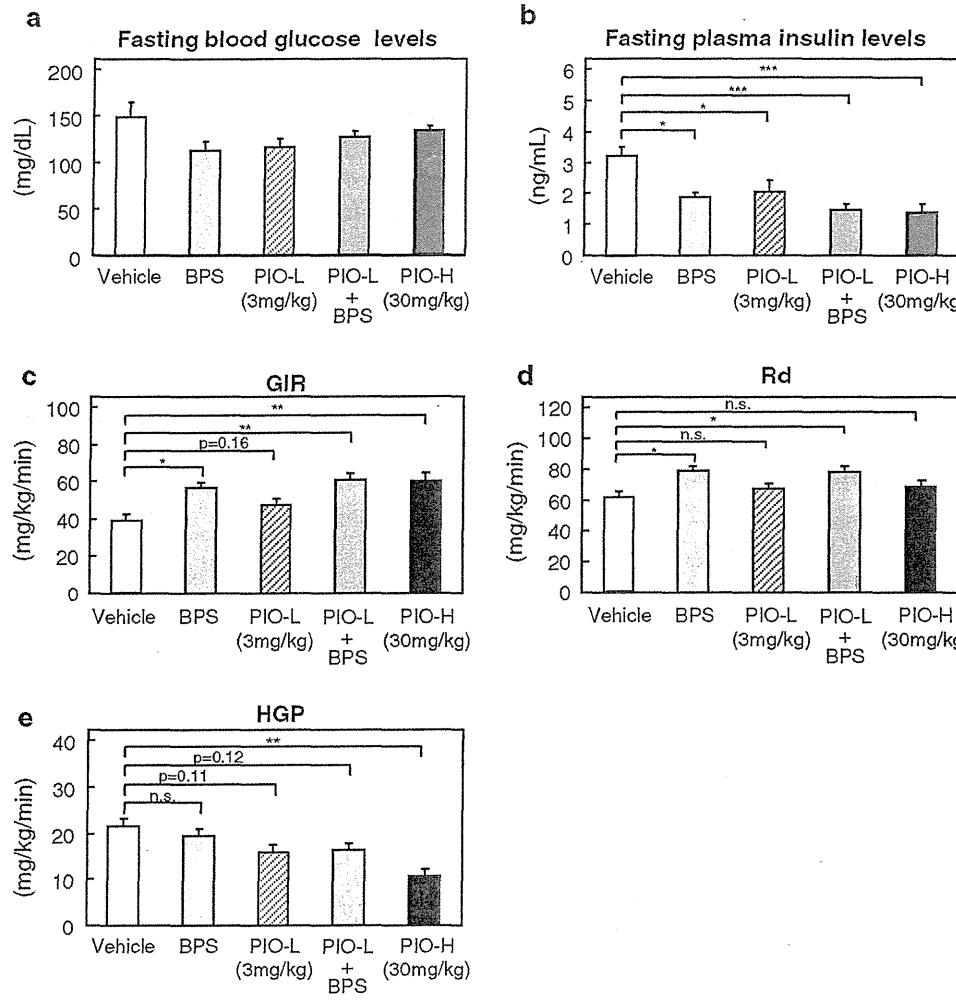

Fig. 2 Combined PIO-L plus BPS treatment ameliorated insulin resistance in KKAy mice. Fasting blood glucose **(a)** and plasma insulin **(b)** levels in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Glucose infusion rates (GIR) **(c)**, rates of glucose disappearance (Rd) **(d)**, and hepatic glucose production (HGP) **(e)** in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H during the hyperinsulinemic-euglycemic clamp study. Values are mean ± SEM ($n = 4$–9). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.001$. *n.s.* not significant

Combined PIO-L plus BPS treatment improved the glucose intolerance in KKAy mice

In the random-fed state, blood glucose levels in the PIO-H- or PIO-L plus BPS-treated KKAy mice were significantly lower as compared with the levels in the vehicle-treated KKAy mice, while the blood glucose levels in latter groups were not significantly different from the value in the vehicle-treated animals (Fig. 3a). Consistent with these data, a significant decrease in the blood glucose levels after glucose loading during the oral glucose tolerance test (OGTT) was observed in the animals treated with PIO-H or PIO-L plus BPS, whereas PIO-L or BPS treatment produced no significant lowering of the blood glucose (Fig. 3b).

Plasma adiponectin levels were significantly correlated with the hepatic glucose production

Since adiponectin production induced by TZD treatment has been reported to improve insulin resistance [4, 5], we next measured the plasma adiponectin levels. Plasma adiponectin levels in the PIO-L, PIO-L plus BPS, and PIO-H treatment groups were significantly increased as compared with the values in the vehicle treatment group, but remained unchanged in the BPS treatment group (Fig. 4a). Furthermore, the plasma adiponectin levels in the PIO-H-treated KKAy mice were significantly higher than those in the BPS-, PIO-L-, or PIO-L plus BPS-treated KKAy mice (Fig. 4a). We next investigated whether the increased plasma adiponectin levels might be correlated with improvement of insulin resistance in KKAy mice. Plasma adiponectin levels and the HGP, but not the Rd, were negatively correlated, suggesting that the increase in plasma adiponectin levels induced by PIO treatment contributed to the improvement of the hepatic, but not the skeletal muscle insulin resistance (Fig. 4b, c).

Combined PIO-L plus BPS treatment decreased the plasma TG levels in KKAy mice

Plasma triglyceride (TG) levels were significantly reduced following PIO-L plus BPS treatment, although no such decrease was observed following treatment with BPS, PIO-L, or PIO-H (Fig. 5a). No significant differences in the

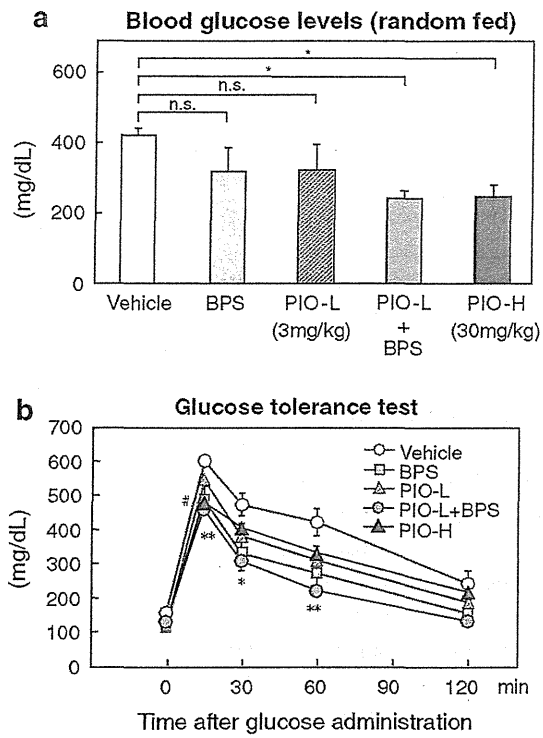

Fig. 3 Combined PIO-L plus BPS treatment ameliorated glycemic control in KKAy mice. **a** Blood glucose levels in random-fed KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. **b** Glucose tolerance test results in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Values are mean ± SEM ($n = 5$–9). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ (in PIO-L plus BPS), $^{\#}p \leq 0.05$ (in PIO-H) compared to vehicle treatment. *n.s.* not significant

plasma-free fatty acid (FFA) or total cholesterol (T-chol) levels were noted among the five treatment groups in this study (Fig. 5b, c).

Discussion

In this study, we demonstrated that PIO-L plus BPS treatment tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance, producing a similar degree of improvement of hyperglycemia as that observed following PIO-H treatment, but without the body weight gain observed in the PIO-H-treated mice. PIO treatment increased the plasma adiponectin levels in a dose-dependent manner, which may have contributed to improvement in the insulin resistance in the liver. On the other hand, BPS treatment ameliorated the skeletal muscle insulin resistance, but not the hepatic insulin resistance. These data suggest that combined PIO-L plus BPS treatment may be a safe and effective anti-diabetic treatment strategy in patients with type 2 diabetes.

In our previous study, low-dose PIO treatment significantly improved the insulin resistance and ameliorated

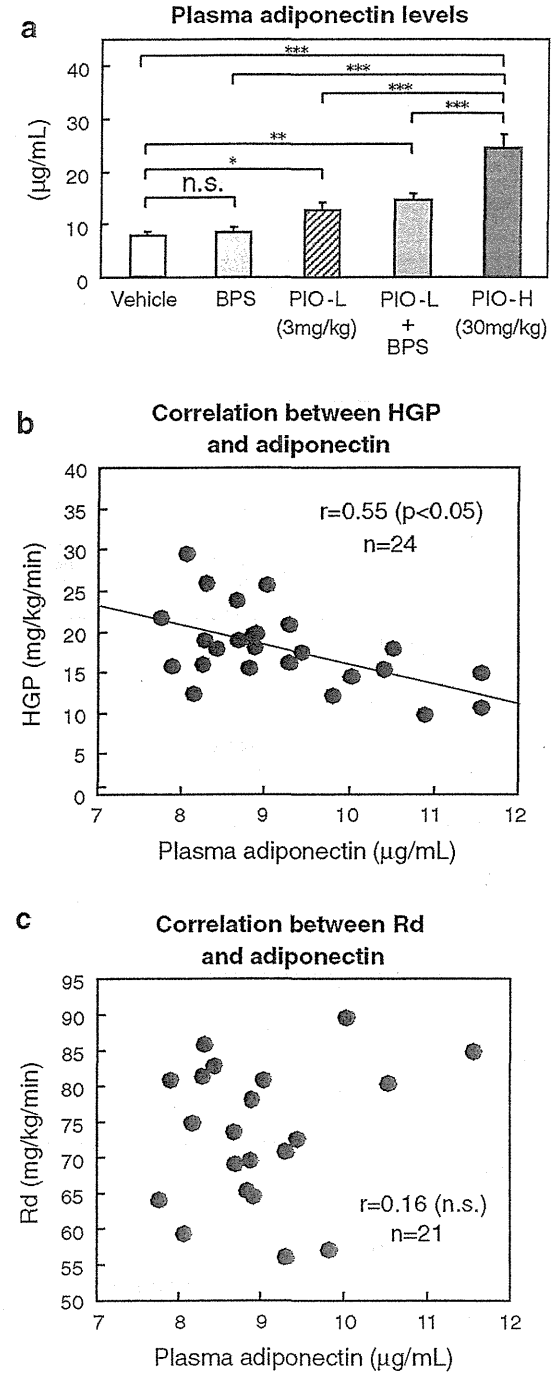

Fig. 4 Plasma adiponectin levels were significantly correlated with hepatic glucose production. **a** Plasma adiponectin levels in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Correlation between the plasma adiponectin levels and hepatic glucose production (HGP) (**b**) and rates of glucose disappearance (Rd) (**c**). Values are mean ± SEM ($n = 5$–10). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *n.s.* not significant

diabetes in an adiponectin-dependent manner in ob/ob mice. The increase in the plasma adiponectin levels following low-dose PIO treatment was attributed to a decrease in glucose production and increase in AMP-activated

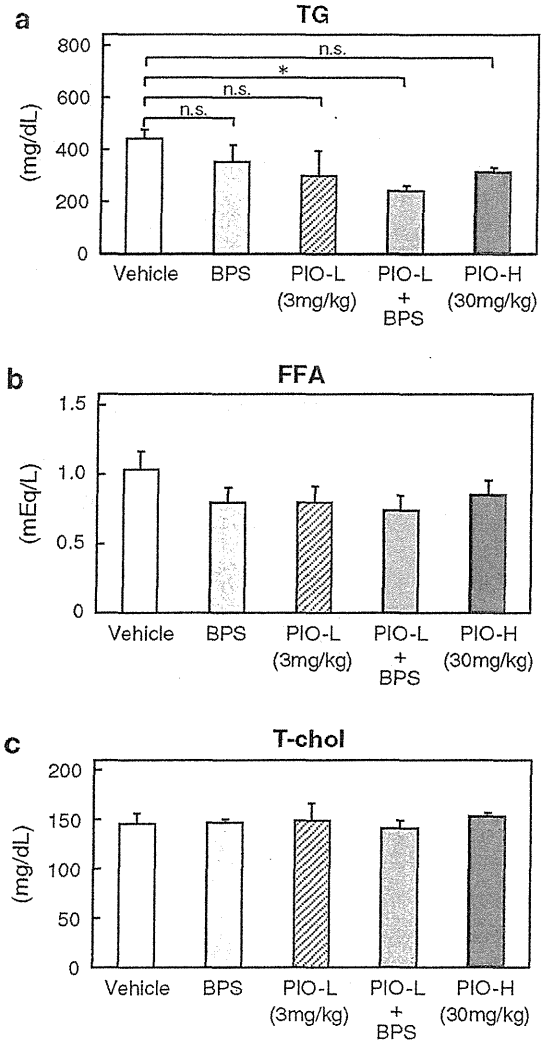

Fig. 5 Combined PIO-L plus BPS treatment decreased plasma TG in KKAy mice. Plasma triglyceride (TG) (**a**), free fatty acid (FFA) (**b**), and total cholesterol (T-chol) (**c**) levels in KKAy mice treated with vehicle, BPS, PIO-L, PIO-L plus BPS, or PIO-H. Values are mean ± SEM ($n = 5$–9). *$p < 0.05$. *n.s.* not significant

protein kinase (AMPK) activation in the liver [10]. Consistent with these data [10], PIO-L treatment in this study increased the plasma adiponectin levels and tended to decrease the HGP in KKAy mice. The plasma adiponectin concentrations were closely related to the HGP (Fig. 4b). These data suggest that the amelioration of hepatic insulin resistance induced by PIO treatment in KKAy mice was mediated, at least in part, via an increase in the plasma adiponectin levels. On the other hand, PIO-H treatment has been shown to ameliorate skeletal muscle insulin resistance in ob/ob mice in an adiponectin-independent manner, accompanied by reduced plasma TG and FFA levels [10]. Since KKAy mice are known to show greater severities of hyperlipidemia than ob/ob mice [19], it is speculated that the plasma TG and FFA levels in the PIO-H-treated KKAy mice were not sufficiently decreased, which may be the reason why the PIO-H treatment failed to improve the skeletal muscle insulin resistance in the KKAy mice.

Skeletal muscle is one of the major target organs of insulin actions and plays an essential role in insulin-induced glucose uptake, giving it a central role in the development of insulin resistance. We recently demonstrated that impaired insulin signaling in the endothelial cells, with reduction of insulin-induced eNOS phosphorylation, reduces insulin-induced glucose uptake by the skeletal muscle, decreased capillary recruitment, and decreased interstitial insulin concentrations in the skeletal muscle [17]. We found that in the HF diet-fed mice, BPS treatment significantly restored the eNOS mRNA and protein expression levels and also the insulin-induced phosphorylation of eNOS to levels similar to those observed in the saline-treated normal chow-fed mice. Restoration of the insulin-induced eNOS phosphorylation in the endothelial cells by BPS treatment also restored the insulin-induced capillary recruitment and interstitial insulin concentrations, resulting in improvement of the skeletal muscle glucose uptake in the HF diet-fed obese mice [17]. Therefore, in the present study also, BPS treatment might have improved the skeletal muscle insulin resistance in KKAy mice via these same mechanisms. Similarly, a significant increase of the insulin-stimulated glucose uptake following infusion of iloprost, a PGI_2 analog, has been reported during a hyperinsulinemic-euglycemic clamp study in subjects with type 2 diabetes [20]. Thus, PGI_2 analogs, including BPS, may serve as novel agents for the treatment of patients with type 2 diabetes with skeletal muscle insulin resistance.

Currently, many patients with type 2 diabetes are receiving treatment with TZDs in combination with other antidiabetic agents. Roy et al. [21] showed that low-dose rosiglitazone (1 mg/kg/day) administered with a DPP4 inhibitor (vildagliptin) yielded similar efficacy to that of high-dose rosiglitazone (10 mg/kg/day) treatment in respect to lowering the blood glucose levels without producing any significant increase in the body weight in obese diabetic db/db mice. These data suggest that low-dose TZD administration in combination with other anti-diabetic agents may also be sufficiently effective for lowering the blood glucose levels, without producing any clinically relevant adverse events [22]. BPS may be one such suitable candidate for administration in combination with low-dose TZDs.

In conclusion, combined PIO-L plus BPS treatment tended to improve the hepatic insulin resistance and significantly improved the skeletal muscle insulin resistance, thereby improving glucose intolerance in KKAy mice to a degree similar to that observed following PIO-H treatment, without causing any body weight gain. This combination therapy therefore appears to be safe and effective for patients with type 2 diabetes mellitus.

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Conflict of interest The authors do not have any conflict of interests to declare.

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