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Amelioration of Hyperglycemia and Metabolic Syndromes in Type 2 Diabetic KKA^{y} Mice by Poly(γ-glutamic acid)oxovanadium(IV) Complex

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Recently, we found that poly(γ-glutamic acid)oxovanadium(IV) complex (VO(γ-pga)) exhibits a potent antidiabetic activity in streptozotocin (STZ)-induced type 1 diabetic mice. This result prompted us to examine its ability to treat the type 2 diabetic model KKA^{y} mice with insulin resistance. We studied the in vivo antidiabetic activity of VO(γ-pga), compared with that of vanadium(IV) oxide sulfate (VS) as control. Both compounds were orally administered at doses of 5–10 mg (0.1–0.2 mmol) V kg^{-1} body mass to the KKA^{y} mice for 30 days. VO(γ-pga) normalized the hyperglycemia within 21 days, whereas VS lowered the blood glucose concentration only by a small degree. In addition, the glucose intolerance, HbA_{1c} level, hyperinsulinemia, hypercholesterolemia, and hyperleptinemia were significantly improved in VO(γ-pga)-treated KKA^{y} mice compared with those treated with VS. Based on these observations, VO(γ-pga) is proposed to be the first orally active oxovanadium(IV)-polymer complex for the efficacious treatment of not only type 2 diabetes but also metabolic syndrome in animals.

Introduction

Type 2 diabetes mellitus (DM)—known as adult-onset diabetes, maturity-onset diabetes, or noninsulin dependent diabetes mellitus (NIDDM)—is due to a combination of defective insulin secretion and defective responsiveness to insulin (often termed insulin resistance or reduced insulin sensitivity), almost certainly involving the insulin receptor in cell membranes.[1,2] The number of patients suffering from type 2 DM is rapidly reaching epidemic levels in several countries. In the early stages, hyperglycemia in type 2 DM can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver, but as the disease progresses the impairment of insulin secretion worsens and therapeutic replacement of insulin often becomes necessary because of the high blood glucose levels from uncontrolled diabetes can cause serious long-term health problems with virtually every system in the body.[3,4] Eventually, they may cause damage to the insulin producing beta cells of the pancreas and some other serious complications of diabetes, which include heart disease, high blood pressure, nerve damage, and kidney failure.[5,6] Several type of therapeutics, such as sulfonylureas, metformin, thiazolidinediones, and meglitinides have been developed for type 2 diabetes.[7–9] However, they have limited efficacy and tolerability and occasionally cause severe side effects.[10,11] To eliminate such undesirable defects, creation and development of new therapeutics to replace insulin injections and synthetic therapeutics are needed to improve the lives of diabetic patients. Vanadium compounds are known to have insulin-mimetic and/or enhancing effects both in vitro and in vivo.[12–14] Several reports have shown that vanadium compounds improve not only hyperglycemia in human subjects and animal models of type 1 diabetes but also glucose homeostasis in genetic obesity, hyperinsulinemia, and insulin resistance in type 2 diabetes.[15–17] The KKA^{y} mouse is known to serve as an excellent model that closely resembles obesity-linked type 2 diabetes in humans. The KKA^{y} mouse expresses several disorders such as hyperglycemia, insulin resistance, hyperleptinemia, hyperinsulinemia, dyslipidemia, obesity, hyperlipidema, and hypertension within a single individual.[18,19] The antidiabetic activity of several vanadyl complexes such as bis(6-methylpicolinato)oxovanadium(IV) complex (VO(6mpa)$_2$), [*meso*-tetrakis(4-sulfonatophenyl)porphyrinato]oxovanadium(IV)(4−) complex (VO(tpps)), and bis(allixinato)oxovanadium(IV)complex, (VO(alx)$_2$) has been evaluated in KKA^{y} mice.[20–22] However, oxovanadium(IV) complexes using polymer as ligand have not been applied yet in any of the type 2 diabetic animal models. We recently found that poly(γ-glutamic acid)oxovanadium(IV) complex (VO(γ-pga)) (Figure 1) exhibits hypoglycemic effects in the streptozotocin (STZ)-induced type 1 diabetic model.[23,24] The complex normalized the hyperglycemia in STZ

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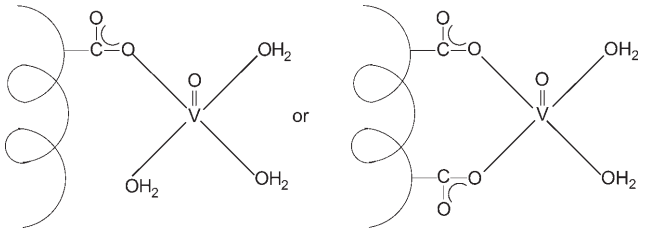

Figure 1. Structure of VO(γ-pga).

mice when it was given orally at doses of 5–10 mg V kg^{-1} body mass for 16–28 days. We then evaluated the effect of oral administration of VO(γ-pga) in obesity-linked type 2 diabetic KKA^{y} mice. Herein, we report the first example of VO(γ-pga) that ameliorates diabetes and metabolic syndromes in type 2 diabetic animals through enhancement of not only insulin sensitivity but also leptin sensitivity.

Results and Discussion

Blood glucose normalization

Previously, we found that VO(γ-pga) is the first orally potent insulin-mimetic oxovanadium(IV)-polymer complex for treating STZ mice.[23,24] We then examined the antidiabetic effects of VO(γ-pga) in KKA^{y} mice upon oral administration for 30 days and compared them with those of the control agent VS. Figure 2a illustrates the change in the blood glucose levels in the saline-treated nondiabetic C57BL/6J mice (Group 1) and the KKA^{y} mice treated with saline alone (Group 2), VS in saline (Group 3), and VO(γ-pga) in saline (Group 4) at doses in the range of 5–10 mg (0.1–0.2 mmol) V kg^{-1} body mass. The blood glucose concentration in the saline-treated KKA^{y} mice (Group 2) was significantly higher than that of the saline-treated nondiabetic C57BL/6J mice (Group 1) throughout the study. Administration of VS at a dose of 5 mg (0.1 mmol) V kg^{-1} body mass for the first 5 days did not result in a decreased blood glucose level in the KKA^{y} mice from Group 3. An increase in the dose to 10 mg (0.2 mmol) V kg^{-1} body mass for the following 25 days also did not result in a significantly lowered blood glucose level in comparison with that of saline-treated KKA^{y} mice. On the other hand, when VO(γ-pga) was administered at a dose of 5 mg (0.1 mmol) V kg^{-1} body mass, the blood glucose level in the KKA^{y} mice (Group 4) was rapidly lowered after 2 days. The same dosage of the complex was then maintained for the following 3 days. However, the blood glucose level was observed to be at approximately 350 mg dL^{-1} (19.4 mM) for the first 5 days. The dose of the complex was then increased to 10 mg (0.2 mmol) V kg^{-1} body mass for the following 25 days. After such an adjustment, the blood glucose level gradually lowered and remained at approximately 200 mg dL^{-1} (10 mM) for the last 11 days; this value was close to the blood glucose levels in the nondiabetic C57BL/6J mice (Figure 2a). Moreover, there was no significant difference between the blood glucose concentrations of the VO(γ-pga)-treated KKA^{y} mice and the saline-treated nondiabetic C57BL/6J mice for the last 10 days (Figure 2a). The blood glucose level remained approximately 300 mg dL^{-1} (16.7 mM) in the KKA^{y} mice after oral treatment with VO(6mpa)$_2$ suspension in 5% acacia at a dose of 5 mg (0.1 mmol) V kg^{-1} body mass for the first 2 days and then 10 mg (0.2 mmol) V kg^{-1} body mass for the following 12 days.[20] These results demonstrated that, following chronic oral administration, VO(γ-pga) exhibits a higher normoglycemic activity in KKA^{y} mice than VS and VO(6mpa)$_2$.[20] The HbA$_{1c}$ concentration in VO(γ-pga)-treated KKA^{y} mice decreased signifi-

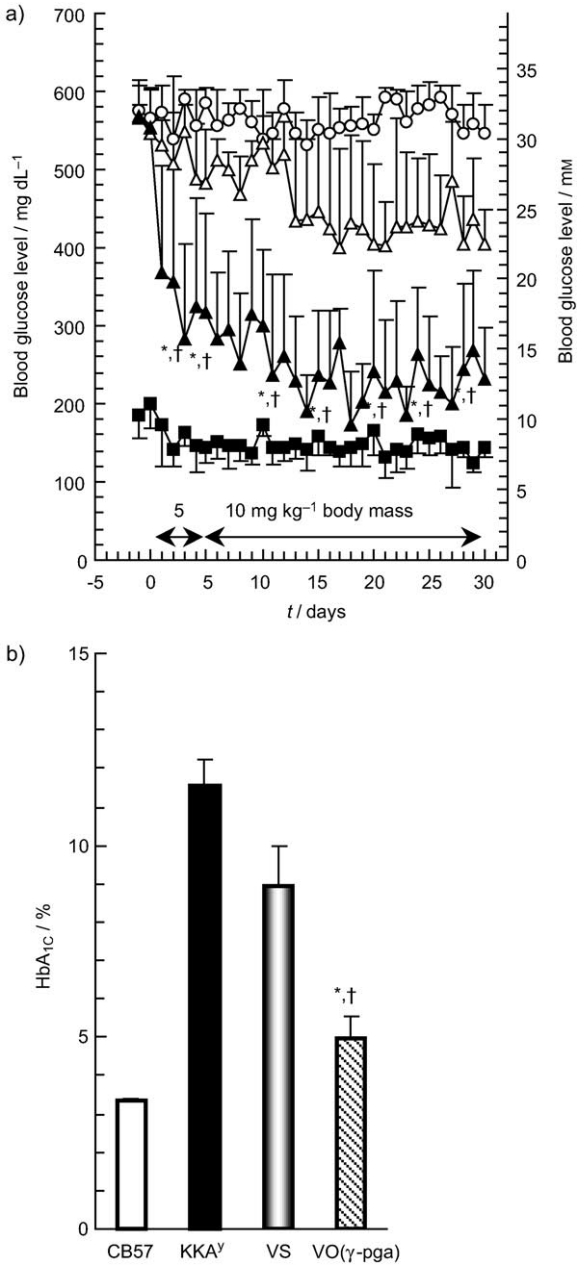

Figure 2. Effects of VO(γ-pga) on blood glucose and HbA$_{1c}$ levels in KKA^{y} mice. a) Changes in blood glucose levels in saline-treated nondiabetic CB57/6J mice (■, $n=5$) and type 2 diabetic KKA^{y} mice treated with saline (○, $n=5$), VS (△, $n=5$), and VO(γ-pga) (▲, $n=7$) at doses of 5–10 mg (0.1–0.2 mmol) V kg^{-1} body mass orally for 30 days. b) Changes in HbA$_{1c}$ levels after VO(γ-pga) treatment for 30 days as described in Figure 2a. Significance: $^{*}p < 0.01$ versus saline-treated KKA^{y} mice; $^{\dagger}p < 0.01$, versus VS.

cantly compared with that in saline-treated KKA^{y} mice (Figure 2b). On the other hand, VS moderately improved HbA_{1c} concentration in KKA^{y} mice. Similar results were observed when STZ mice received either VO(γ-pga) at doses of 5–10 mg (0.1–0.2 mmol) V kg^{-1} body mass ($HbA_{1c} = 6.4 \pm 0.5\,\%$) or saline alone ($HbA_{1c} = 9.5 \pm 1.0\,\%$) for 16 days.[23] These results indicate that VO(γ-pga) treatment provides glycemic control not only in type 1 diabetic mice[23] but also in type 2 diabetic KKA^{y} mice. In the OGTT after the treatment period, the blood glucose level of the saline-treated KKA^{y} mice increased to a maximal concentration of 471 mg dL^{-1} (26.2 mM) at 30 min after glucose loading, whereas the blood elevation in the VO(γ-pga)-treated KKA^{y} mice was almost equivalent to that of the normal mice (Figure 3a). The plasma hyperinsulinemia in KKA^{y} mice was also normalized by the VO(γ-pga) treatment (Figure 3b). These results indicate that the VO(γ-pga) treatment ameliorates the insulin resistance and glucose intolerance in KKA^{y} mice. The pathogenesis of type 2 diabetes is characterized by insulin resistance in insulin-targeting tissues such as the muscle, fat, liver, and brain. The action of insulin is initiated by its binding with the α-subunit of the insulin receptor; this enhances autophosphorylation, which in turn stimulates the tyrosine kinase in the β-subunit of insulin receptor (IRK).[25] Subsequently, the IRK phosphorylates the insulin receptor substrate (IRS). Following these reactions, the signal information is transferred to the downstream molecules such as phosphatidylinositol 3-kinase (PI3-K), protein kinase B (PKB), and GLUT-4. Consequently, several reactions (for example, glucose uptake or gluconeogenesis) are enhanced in the insulin responsive tissues. The insulin signal pathway has been shown to be negatively regulated by phosphotyrosine phosphatases (PTPs) that is, PTP1B.[26] The enhanced PTP1B activity has been shown in patients or animals with obesity, insulin resistance, and type 2 diabetes.[27,28] Therefore, the inhibition of PTP1B may enhance the impaired insulin signal transduction in type 2 diabetes.[29] In fact, treatments with PTP1B inhibitor or antisense in KKA^{y} mice normalized the glucose and insulin levels in type 2 diabetic mice with obesity.[29,30] Vanadium compounds have been considered to enhance insulin sensitivity and glucose uptake by inhibiting the PTP1B activity in type 2 diabetic animals.[29,31,32] The oxovanadium(IV) ion and its complexes have also been revealed to act on at least four sites involving an insulin-dependent signal transduction system, glucose transporter, and phosphodiesterase.[33] Therefore, VO(γ-pga) might improve insulin resistance either by inhibiting PTP1B activity or by acting at several sites involved in insulin-dependent signal transduction system, glucose transporter, and phosphodiesterase in KKA^{y} mice.

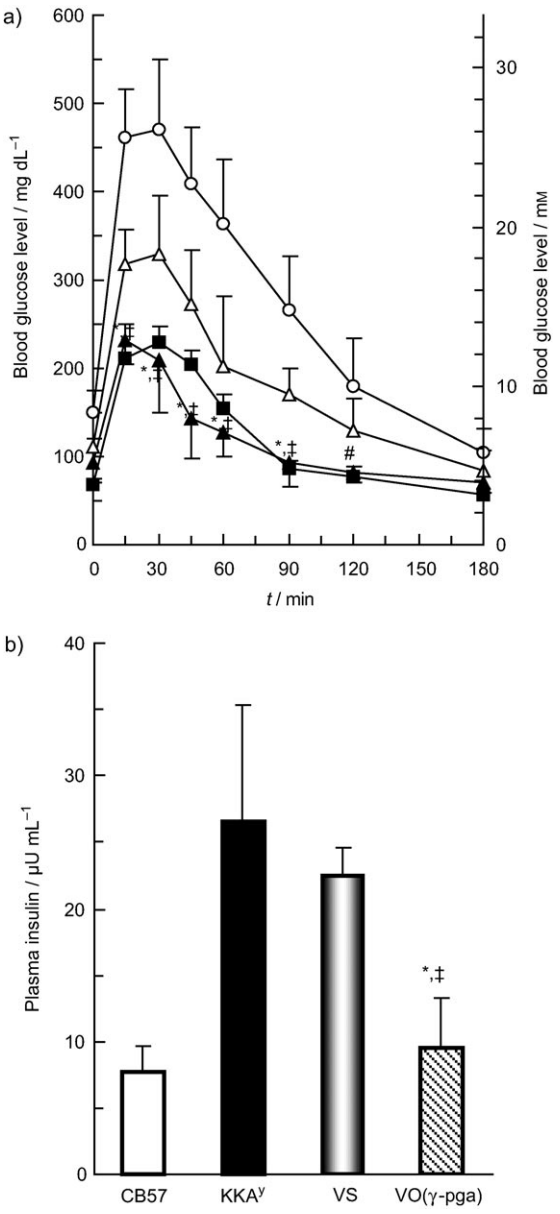

Figure 3. Effects of VO(γ-pga) on glucose tolerance and plasma insulin level in KKA^{y} mice. a) Changes in blood glucose levels in saline-treated nondiabetic CB57/6J mice (■, $n = 5$) and in type 2 diabetic KKA^{y} mice during oral glucose tolerance test (OGTT) after treatment with saline (○, $n = 5$), VS (△, $n = 5$), and VO(γ-pga) (▲, $n = 7$) for 30 days. OGTT tests were performed on mice that had fasted for 12 h, and then they were given oral glucose solution at a dose of 1 g kg^{-1} body mass. b) Plasma insulin levels after VO(γ-pga) treatment for 30 days as described in Figure 2a. Each symbol is expressed as the mean value ± SD ($n = 5$–7). Significance: $^{*}p < 0.01$, $^{\#}p < 0.05$ versus saline-treated KKA^{y} mice; $^{\ddagger}p < 0.05$ versus VS.

VO(γ-pga) alleviates obesity in KKA^{y} mice by improving leptin resistance

Leptin, which is primarily synthesized and secreted from white adipose tissue, is a hormonal protein that regulates glucose metabolism and insulin sensitivity via leptin receptors in the brain and peripheral tissues.[34] The discovery of leptin has stimulated research on obesity because this adipocytokine has been shown to strongly relate to the developing type 2 diabetes.[34] Hyperleptinemia was observed in KKA^{y} mice; it was positively related with degree of adiposity (Figure 4a and b), indicating a state of leptin resistance in obesity. VO(γ-pga) reduced the high plasma leptin levels in KKA^{y} mice (Figure 4a), suggesting enhancement of leptin sensitivity. The improved leptin sensitivity suppressed the excessive food intake and the weight of epididymal fat pads without affecting the body weight change (Figure 4b–d). These results indicate that VO(γ-pga) amelio-

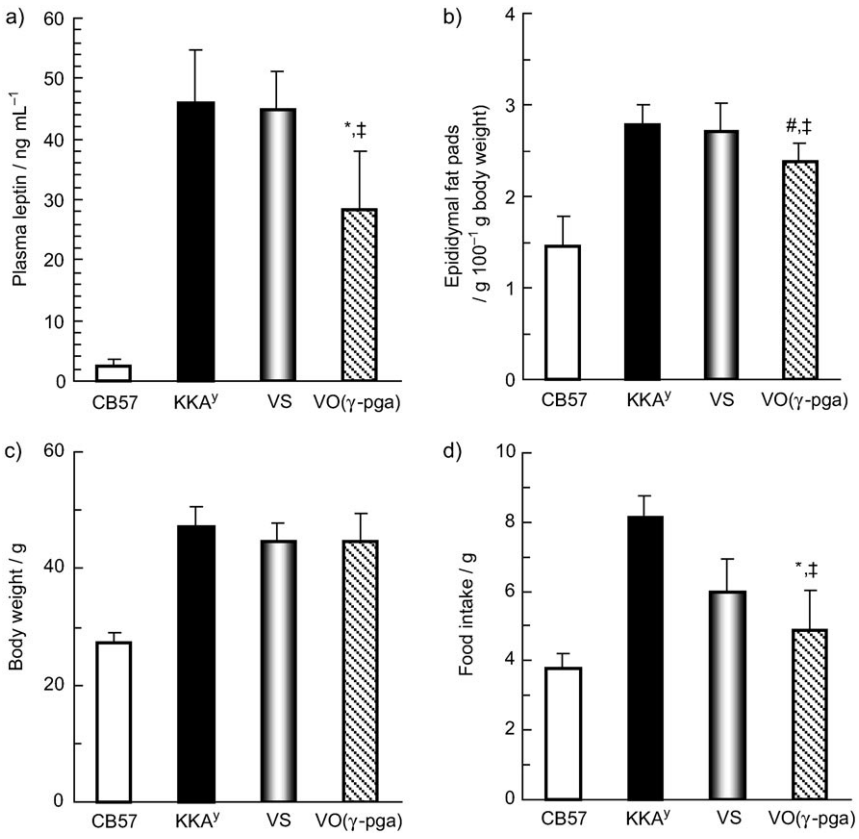

Figure 4. Effects of VO(γ-pga) on obesity in KKA^{y} mice. a) Plasma leptin, b) epididymal fat weight, c) body weight, and d) food intake after VO(γ-pga) treatment as described in Figure 2 a. Data are expressed as the mean value ± SD ($n = 5$–7). Significance: $^{*}p < 0.01$, $^{\#}p < 0.05$ versus saline-treated KKA^{y} mice; $^{+}p < 0.05$ versus VS.

rates obesity by enhancing leptin sensitivity in KKA^{y} mice. This supports the previous finding that vanadium compounds treat hyperleptinemia in KKA^{y} mice.[21,22]

Plasma parameters

The levels of UN, GPT, GOT, TG, TCHO, and FFA in the plasma of the C57BL/6J and KKA^{y} mice are summarized in Table 1. The plasma UN level (21.6 ± 4.1 mg dL^{-1}), which indicates the degree of renal disturbance, was lowered significantly in the VO(γ-pga)-treated KKA^{y} mice compared with that in the saline-treated (33.2 ± 4.2 mg dL^{-1}; $p < 0.01$) and VS-treated (30.7 ± 6.4 mg dL^{-1}; $p < 0.05$) KKA^{y} mice. Moreover, the plasma UN level in the CB57/6J mice was determined to be 25.9 ± 1.2 mg dL^{-1}. The GOT (53 ± 3 U L^{-1}) and GPT (21 ± 2 U L^{-1}) levels in the VO(γ-pga)-treated KKA^{y} mice, which show the degree of liver disturbance, were also significantly suppressed compared with those in the saline-treated KKA^{y} mice. The plasma GOT and GPT levels in the CB57/6J mice were 41 ± 16 U L^{-1} and 18 ± 2 U L^{-1}, respectively. The plasma TCHO level (114 ± 8 mg dL^{-1}) in the KKA^{y} mice following treatment with VO(γ-pga) was lower compared with that in the KKA^{y} mice treated with saline alone (154 ± 21 mg dL^{-1}), indicating that cholesterol metabolism was improved by the treatment with VO(γ-pga). This supports the previous results that vanadium compounds treat hypercholesterolemia in KKA^{y} mice.[21,22] The plasma TG level was lowered (187 ± 31 mg dL^{-1}) in the VO(γ-pga)-treated KKA^{y} mice compared with the saline-treated KKA^{y} mice (220 ± 69 mg dL^{-1}). On the other hand, the plasma TG level (220 ± 27 mg dL^{-1}) in the VS-treated KKA^{y} mice was not improved compared with the saline-treated KKA^{y} mice. The plasma TG level in the CB57/6J mice was estimated to be 85 ± 8 mg dL^{-1}. In contrast to the present results, the plasma TG level was unchanged in diabetic animals after treatment with VO(alx)$_2$.[22] However, it was reported that the chronic oral administration of VO(mal)$_2$ reduced plasma TG levels in Zucker diabetic fatty rats.[35] Diabetes is often associated with hyperlipidemia, that is, elevated levels of TG and nonesterified fatty acids. Therefore, the plasma TG lowering effect of VO(γ-pga) may be beneficial to diabetic patients. The plasma FFA levels were almost the same in all groups of KKA^{y} mice (Table 1). Moreover, the plasma UN,

Table 1. Improvement in plasma biochemical parameters of KKA^{y} mice after the treatment with VO(γ-pga) for 30 days.

Treatment group	Dose [mg V kg^{-1}] [mmol V kg^{-1}]	n	UN [mg dL^{-1}]	GOT [U L^{-1}]	GPT [U L^{-1}]	TG [mg dL^{-1}]	TCHO [mg dL^{-1}]	FFA [mEq L^{-1}]
CB57/6J		5	25.9 ± 1.2	41 ± 16	18 ± 2	85 ± 8	102 ± 7	0.904 ± 0.101
KKA^{y}		5	33.2 ± 4.2	58 ± 4	29 ± 5	220 ± 69	154 ± 21	1.725 ± 0.151
KKA^{y}-VS	5–10 (0.1–0.2)	5	30.7 ± 6.4	65 ± 5	25 ± 4	220 ± 27	139 ± 12	1.693 ± 0.061
KKA^{y}-VO(γ-pga)	5–10 (0.1–0.2)	7	21.6 ± 4.1$^{*,+}$	53 ± 3^{+}	21 ± 2$^{\#}$	187 ± 31	114 ± 8$^{\#,\dagger}$	1.705 ± 0.151

Significance: $^{*}p < 0.01$, $^{\#}p < 0.05$ versus saline-treated KKA^{y} mice; $^{\dagger}p < 0.01$, $^{+}p < 0.05$ versus VS.

GPT, GOT, TG, and TCHO levels in the KKA^y mice treated with either saline alone or with VS dissolved in saline were quite similar indicating that no plasma parameter in KKA^y mice are improved by VS. In conclusion, VO(γ-pga) is the first example of an orally active oxovanadium(IV)-polymer complex with a VO(O_4) coordination environment that is useful for treating type 1 diabetic STZ mice[23,24] and type 2 diabetic KKA^y mice. In addition, VO(γ-pga) is expected to have potential with regard to the treatment of not only type 2 diabetes but also metabolic syndromes in animals.

Experimental Section

Materials

VO(γ-pga) was prepared in a saline solution (pH 3) as reported previously.[23] Poly(γ-glutamic acid), (γ-pga), with a D:L enantiomeric mixture having the average molecular weight 5.0×10^5 Da was a product of BioLeaders Japan Corporation (Osaka, Japan). The polymer was used without further purification. Vanadium(IV) oxide sulfate *n*-hydrate ($VOSO_4{\cdot}nH_2O$) obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan) was standardized complexometrically with EDTA (ethylenediamine-*N*,*N*,*N*,*N*-tetraacetic acid) and ascertained to be a trihydrate (VS). All other reagents were commercially available in the highest grade of purity and were used without further purification. The aqueous solution of γ-pga was prepared in deionized distilled water by adding μL amounts of 5 M NaOH.

Animals

Male nondiabetic C57BL/6J mice (5 weeks old, 17–19 g) and male KKA^y mice (5 weeks old, 25–27 g) were obtained from CLEA Japan, Inc. (Osaka, Japan), and were used for in vivo study when they were 12 weeks old. KKA^y mice, which are obtained by crossing glucose-intolerant black KK female mice with yellow male obese A^y mice, are characterized by hyperphagia due to leptin resistance, followed by obesity, and the development of hyperleptinemia, insulin resistance, hyperinsulinemia, diabetes, dyslipidemia, and hypertension after approximately 8 weeks of age.[18,19] The C57BL/6J mice were generally employed as a nondiabetic control for the KKA^y mice. All animals were individually housed in a cage and maintained on a 12 h light-dark cycle in a temperature-controlled central animal room. All mice were allowed free access to solid food (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water. All animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and performed according to the guidelines for animal experimentation developed by KPU.

In vivo evaluation in KKA^y mice

The mice were divided into the following four groups: group 1, nondiabetic C57BL/6J mice ($n=5$) that were orally administered saline alone; group 2, control KKA^y mice ($n=5$) that were orally administered saline alone; group 3, KKA^y mice ($n=5$) that were orally administered VS dissolved in saline; group 4, KKA^y mice ($n=7$) that were orally administered VO(γ-pga) prepared in saline. VS and VO(γ-pga) prepared in saline solution (pH 3) were administered to the mice from groups 3 and 4, respectively, at doses of 5 mg (0.1 mmol) V kg^{-1} body mass for the first 5 days and then 10 mg (0.2 mmol) V kg^{-1} body mass for the following 25 days. These agents were orally administered to each mouse for 30 days at about 11.00 a.m. after the determination of their blood glucose level. The blood sample required for the daily analyses of glucose levels was collected from the tail vein of each mouse, and the blood glucose level was measured using a GLUCOCARD (ARKRAY Inc., Kyoto, Japan). The body mass of the mice, which were allowed free access to solid food (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water, was measured daily before the administration of the saline and vanadium(IV) compounds. The intake of solid food and drinking water by each mouse was measured daily throughout the experiment before the administration of the saline and vanadium(IV) compounds. Following VO(γ-pga) treatment for 30 days, the glycosylated hemoglobin (HbA_{1c}) levels in the blood collected from the tail veins of the mice were measured using a DCA 2000 analyzer (Bayer Corp., Tokyo, Japan).

Oral glucose tolerance test

After VO(γ-pga) treatment for 30 days, oral glucose tolerance tests (OGTT) were performed. All mice were fasted for 12 h, and glucose at a dose of 1 g kg^{-1} body mass was administered orally. Blood samples were collected from the tail veins at 0, 15, 30, 45, 60, 90, 120, and 180 min after the glucose administration. The blood glucose levels were measured using the GLUCOCARD as described above.

Blood sampling and assay

After the oral administration of VO(γ-pga) for 30 days, the blood samples were collected by orbital exsanguination of the mice under ether anaesthesia by using heparinized tools; the samples were centrifuged at 5000 rpm for 10 min at 4 °C. The resultant plasma was used for the analyses of biochemical parameters. Plasma urea nitrogen (UN), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), triglycerides (TGs), and total cholesterol (TCHO) were determined by using a Fuji Dry Chem analyzer (Fuji Medical Co. Ltd, Tokyo, Japan). Plasma insulin and free fatty acids (FFAs) levels were measured using a Glazyme insulin-EIA test (Sanyo Kasei Co., Kyoto, Japan) and a NEFA C-test (Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively. The plasma leptin levels were measured using a Quantikine Mouse Leptin Immunoassay kit from R&D Systems, Inc. (Minneapolis, MN, USA).

Statistical analysis

All experimental results are expressed as mean ± standard deviation (SD). Statistical analysis was performed by using one-way analysis of variance followed by Tukey-Kramer's multiple-comparison post-hoc tests. Differences were considered to be statistically significant when $p<0.01$ or <0.05.

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Keywords: hyperglycemia · insulin resistance · KKA^y mice · leptin resistance · obesity

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