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Full Length Research Paper

Vanadyl-poly(γ-glutamic acid) complexes as oral therapeutic agents for the treatment of type 1 like diabetic mice

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In developing new insulin-mimetic vanadyl complexes with various ligands including biodegradable polymers, we prepared and characterized three VO(γ-pga) complexes in solution and evaluated their *in vitro* insulin-mimetic activities and *in vivo* anti-diabetic effects in streptozotocin (STZ)-induced type 1 like diabetic mice (STZ-mice). All three VO(γ-pga) complexes normalized the hyperglycemia in STZ-mice within 14 d when administered orally at doses of 10 mg V kg^{-1} body mass for 28 d. In addition, the impaired glucose tolerance, elevated HbA_{1c} levels and metabolic syndromes were significantly improved in VO(γ-pga) complexes-treated STZ-mice relative to those administrated with saline and VS. Vanadium was distributed in the tissues examined in the following decreasing order: bones, liver, muscles, spleen, heart, kidneys, brain, lungs and pancreas. VO(γ-pga) complexes in which γ-pga having average molecular weight 480 – 4700 kDa are promising oral therapeutic agents for the treatment of type 1 diabetic animals.

Key words: Vanadyl-poly(γ-glutamic acid) complex, STZ-mice, drug delivery system, diabetes, hyperglycemia.

INTRODUCTION

The number of patients suffering from diabetes mellitus (DM) is rapidly reaching epidemic levels in several countries. Generally, DM can be divided into two major groups: type 1 and type 2 DM. Briefly, in case of type 1 DM, which results from destruction of the β cells (Robin et al., 2003) the major cause of destruction of the islet cells is autoimmunity (type 1A) and minor cause is idiopathic (type 1B). Type 2 DM results from derangement in β cell secretion of insulin and an inability of the peripheral target tissues to respond to insulin (insulin resistance) (Robin et al., 2003). More importantly, the long-term complications in blood vessels, kidneys, eyes and nerves occur in both types of DM and are the major cause of morbidity and death from DM (Quraishi et al., 2005). Based on its type, the treatment for DM involves either daily injections of exogenous insulin (most common for type 1 DM) or oral administration of hypoglycemic drugs, such as sulfonylureas, metformin, alpha-glucosidase inhibitors, thiazolidinediones and meglitinides and in a combination therapy for type 2 DM (Groop, 1992; Wysowski et al., 2003; Inzucchi, 2002; Karter et al., 2005). However, this approach is not satisfactory for an important number of patients; hence, there have been continued efforts towards developing new hypoglycemic drugs with high potency, but little or no side effects. It has been established that vanadium compounds exert insulin-mimetic and/or enhancing effects both *in vitro* and *in vivo* systems (Sakurai et al., 1990; Mehdi et al., 2006; Thompson and Orvig, 2004; Shechter et al., 2003; Rehder 2003; Crans et al., 2004; Saha et al., 2006). Several reports have documented 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 (Shibuichi et al., 2006; Thompson and Orvig, 2006; Goldfine et al., 2000;

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Cusi et al., 2001). Since the discovery of a novel orally active insulin-mimetic vanadyl-poly(γ-glutamic acid) complex (VO(γ-pga)) for the treatment of streptozotocin (STZ)-induced diabetic mice (STZ-mice) - a type 1 diabetic model - in 2006 (Karmaker et al., 2006) in which the average molecular weight of γ-pga was 500 kDa, the therapeutic potential of vanadyl complexes has been of great interest to us (Saha et al., 2007; Karmaker et al., 2007; Karmaker et al., 2008). Poly(γ-glutamic acid) (γ-pga) is a naturally occurring biodegradable and biocompatible polymer containing side chain of carboxyl groups, it is also nontoxic toward humans and the environment (Saino-Oppermann and Steinbuchel, 2000; Sung et al., 2005). To better define the efficacy of VO(γ-pga) complex, further studies are needed to determine whether this complex is beneficial or detrimental in the treatment of diabetes mellitus. In this study, we have prepared and characterized various VO(γ-pga) complexes in solution using three different types of γ-pga in terms of molecular weight. VO(γ-pga) complexes prepared in solution were used to evaluate their *in vitro* insulin-mimetic activities in isolated rat adipocytes treated with epinephrine and *in vivo* antidiabetic effects in STZ-mice. After 28 days oral administrations of three VO(γ-pga) complexes to STZ-mice, oral glucose tolerance ability, glycosylated hemoglobin (HbA_{1c}) levels, blood pressure, serum parameters and total vanadium concentration in several organs of STZ-mice were measured. Finally, we compared the observed results with those obtained by using vanadyl(IV) sulfate dihydrate (VS) and previously reported VO(γ-pga) complex as positive controls (Karmaker et al., 2006).

MATERIALS AND METHODS

Materials

The samples of γ-pga with a D: L enantiomeric mixture having the average molecular weights 480, 2500 and 4700 kDa were kindly provided from GENOLAC BL Corp. (Osaka, Japan). Three different types of γ-pga samples were denoted by γ-pga1, γ-pga2 and γ-pga3, respectively, according to the increasing order of average molecular weight. These polymers were used without further purification. Vanadyl(IV) sulfate n-hydrate ($VOSO_4 \bullet nH_2O$) obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan) was standardized by complexometrically with ethylenediamine-*N,N,N,N*-tetraacetic acid (EDTA) and ascertained to be a dihydrate ($VOSO_4 \bullet 2H_2O$; VS). Bovine serum albumine (BSA; fraction V), (±)-epinephrine monohydrochloride and collagenase were purchased from Sigma Chemical (Sigma, St. Louis, MO., USA). All other reagents were commercially available in the highest grade of purity and were used without further purification. The solutions of γ-pga were prepared in aqueous solution by adding micro liters amount of 5 M NaOH.

Preparation and characterization of VO(γ-pga) complexes in solution

The VO(γ-pga) complexes were prepared in solution as described previously (Karmaker et al., 2006). Typically, a sample solution at the appropriate pH was prepared by mixing VS (1 M) and γ-pga (1 - 40% w/v) solutions under magnetic stirring. Three different types of VO(γ-pga) complexes were denoted by VO(γ-pga1), VO(γ-pga2) and VO(γ-pga3), respectively, according to the increasing order of average molecular weight of γ-pga. The structures of VO(γ-pga) complexes were characterized by UV-visible and electron spin resonance (ESR) spectra. The most suitable pH is 3 for the VO(γ-pga) complexation. The pH of the reaction mixtures was determined by using HORIBA pH meter M-12 (Japan). The pH of the samples was adjusted by adding micro liter quantities of 5 M NaOH or 5 M HCl. The VO(γ-pga) complexes in solution were used *in vitro* and *in vivo* experiments.

Evaluation of *in vitro* insulin-mimetic activity

Insulin-mimetic activities of the VO(γ-pga) complexes were evaluated by in vitro experiments in which the inhibitory activities of complexes on the release of free fatty acid (FFA) (Nakai et al., 1995) in isolated rat adipocytes treated with epinephrine were estimated and compared with those of VS and previously reported VO(γ-pga) complex as positive controls (Karmaker et al., 2006).

Evaluation of *in vivo* antidiabetic activity

Experimental type 1 like DM was induced in 6-week-old male std: ddY mice, weighing approximately 30 g, by two *i.p.* injections of freshly prepared STZ (100 mg kg^{-1} body mass) in 0.1 M citrate buffer (pH 5) at one week interval. Blood samples for the analysis of glucose level were obtained from tail vein of STZ-mice and the levels were measured using glucose oxidase method with a Glucocard (Arkray, Kyoto, Japan). Diabetic mice with a blood glucose level of 450 - 550 mg dL^{-1} (25 - 30.6 mM) at 4 weeks after the first STZ administration were used for the experiments. In total 36 mice were divided into six experimental groups: saline-treated non-diabetic normal std: ddY mice (n = 6), saline-treated STZ-mice (n = 6) and STZ-mice treated with VS (n = 6), VO(γ-pga1) (n = 6), VO(γ-pga2) (n = 6) and VO(γ-pga3) (n = 6). The VO(γ-pga) complexes and VS were administered daily to the STZ-mice by oral gavages for 28 days at doses 10 mg V kg^{-1} body mass. VO(γ-pga) complexes were freshly prepared in saline solution at pH 3 by mixing adequate amount of VS (1 M) with various γ-pga (2% w/v). The final concentration of vanadyl ion in complexes was 20 mM. The blood sample for the analyses of glucose levels was obtained from the tail vein of each mouse and the blood glucose level was measured with a Glucocard as described earlier. Body mass of non-diabetic normal mice and STZ-mice, which were allowed free access to solid food (MF, Oriental Yeast, Tokyo, Japan) and tap water, were measured daily before the administration of saline, VS and VO(γ-pga) complexes. In addition, intakes of solid food and drinking water in each mouse were checked daily throughout the experiment.

The HbA_{1c} level in the blood obtained from the tail veins of the mice after the treatment with three VO(γ-pga) complexes was determined by using a DCA 2000 system (Bayer, Tokyo, Japan). The systolic blood pressure of the mice was measured by the indirect tail-cuff method using a blood pressure (BP) monitor for rats and mice (MK-2000, Muromachi Kikai, Tokyo, Japan) according to the manufacturer's instructions. After daily oral administration of VS and three VO(γ-pga) complexes for 28 days, an oral glucose tolerance test (OGTT) was performed. The STZ-mice were fasted for 12 h and glucose solution at a dose 1 g kg^{-1} body mass was given orally. Blood samples were obtained from the tail vein at 0, 15, 30, 45, 60, 90, 120 and 180 min after glucose administration. Following the OGTT, blood samples were collected from orbital exsanguinations of the mice under anesthesia with ether and were

centrifuged at 5000 rpm for 10 min at 4°C. The serum samples were thus separated and used for the analysis of urea nitrogen (UN), glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), triglycerides (TG), total cholesterol (TCHO), free fatty acid (FFA) and insulin levels. The serum UN, GPT, GOT, TG and TCHO levels were estimated by using a Fuji Dry Chem analyzer (Fuji Medical Co., Tokyo, Japan). A NEFA C-test and Glazyme insulin-EIA test (Wako Pure Chemical Industries, Osaka, Japan) were used to determine the serum FFA and insulin levels, respectively.

Finally, the mice were sacrificed under ether anesthesia and organs, such as the liver, spleen, pancreas, kidneys, lungs, heart, brain, muscles and bones, were removed and lyophilized. The vanadium concentration was determined by the inductively coupled plasma mass spectrometer (ICP-MS) (Shimadzu ICP-MS8500, Kyoto, Japan). The concentration of vanadium was calculated using a calibration curve at a concentration range of 5 - 100 ppb for the standard vanadium solutions (Wako Pure Chemical Industries Ltd., Osaka, Japan). The detection limit of vanadium concentration was approximately 0.01 ppb. The correlation coefficient of linear regression was r = 0.999 for a total of four metal concentrations. Plasma conditions for ICP-MS were as follows: coolant gas-flow, plasma gas-flow and carrier gas-flow were 7.0, 1.5 and 0.61 L min^{-1}, respectively and the sampling depth was 6 mm.

Statistical analysis

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

RESULTS AND DISCUSSION

Preparation and characterization of VO(γ-pga) complexes

The VO(γ-pga) complexes were prepared in aqueous solution (pH 1.5 - 5) by mixing VS (1 M) and γ-pga (1 - 40% w/v) solutions. The structure of the complexes was determined by the UV-visible absorption and ESR spectra. The physico-chemical parameters of VS and all three VO(γ-pga) complexes are summarized in Table 1.

The absorption maxima of all three VO(γ-pga) complexes in solution (pH 3) were found in the visible range at around 780 nm with a shoulder at 600 nm, whereas free γ-pga absorbed only at the UV-region and VS showed an absorption band at 765 nm with a shoulder at 620 nm. Thus the red shift (15 nm) of the absorption band at around 780 nm and the blue shift (20 nm) of the absorption band at 600 nm in the visible region must be ascribed to transition of electrons associated with the complex formation. The shift of the two typical bands of free VO^{2+} to red (15 nm) and blue (20 nm), respectively, in the complexes also suggest coordination of this metal ion through the carboxylate groups of the γ-pga (Karmaker and Saha, 2008). Moreover, the absorbance intensities at 780 nm of VO(γ-pga) complexes in solution were found to be high at pH 3 among the observed pH at 1.5 - 5 (data not shown), suggesting that the most suitable pH was 3 for all three VO(γ-pga) complexation as observed in our previous study (Karmaker and Saha, 2008).

ESR spectra of VS in aqueous solution (pH 3) exhibited eight lines at room temperature and an anisotropic spectrum at liquid nitrogen temperature (data not shown). On the other hand, VO(γ-pga) complexes in aqueous solution (pH 3) exhibited anisotropic ESR spectra at room and liquid nitrogen temperatures (data not shown), when the complexes were prepared at the ratio of 1:0.8 of VS and γ-pga (final concentration of VS: 1 mM and γ-pga: 40%), indicating the existence of vanadyl species upon the complexation of VO^{2+} with γ-pga (Karmaker and Saha, 2008). Similar phenomenon was observed in the ESR spectrum of 1:0.8 solution of $CuSO_4$ and γ-pga (final concentration of $CuSO_4$: 1 mM and γ-pga: 40%) at room temperature (Karmaker et al., 2007). These results indicate that the VO(γ-pga) complexes are formed by the coordination of VO^{2+} through the side chain carboxylic groups of the γ-pga.

In order to determine the coordination mode of VO(γ-pga) complexes and the optimum pH for the complexation, the measurements of ESR spectra of the complexes were extended over a range of pHs 2 – 5, where the final concentrations of VS (1 mM) and γ-pga (40%) were kept constant. Typical ESR spectra of VO(γ-pga1) complex in aqueous solution as a function of pH at liquid nitrogen temperature are given in Figure 1a. The estimated ESR parameters are tabulated in Table 1. The values of $g_{//}$ and $A_{//}$ were found to be almost constant at pH 2–5 (Table 1), indicating the coordination sphere around VO^{2+} is $VO(O_4)$ as judged from the reference values reported previously (Karmaker and Saha 2008; Sakurai et al., 1990; Rangel et al., 2001; Yasui et al., 2002). On the other hand, the highest signal intensities of all three VO(γ-pga) complexes at liquid nitrogen temperature were observed at pH 3 among the observed pH at 2 – 5, suggesting that the most suitable pH was 3 for the VO(γ-pga) complexation (Karmaker and Saha, 2008). A typical plot of the changes of ESR signal intensity at 290 mT of VO(γ-pga1) complex as a function of pH is shown in Figure 1(b). The hyperfine coupling constants, A_0 and $A_{//}$, of all VO(γ-pga) complexes were also calculated with the application of the so-called additivity relationship (Chasteen, 1981),

$$A_{0,\text{calcd}} = \sum n_i A_{0,i}/4$$

Where i denotes the different types of donor atoms ligated to the equatorial positions of VO^{2+}, n_i = (1 to 4) is the number of donor atoms of type i and $A_{0,i}$ is the observed coupling constant (from model studies) when all four equatorial donor atoms are of type i. The theoretical A_0 and $A_{//}$ values were estimated based on various (O_4) equatorial coordination modes of VO^{2+} in VO(γ-pga) complex. The calculated A_0 and $A_{//}$ values of 102×10^{-4}

Table 1. Physico-chemical parameters of VS and three VO(γ-pga) complexes prepared in aqueous solution.

Sample	Solvent	pH	Visible absorption maxima (nm)	ESR parameters					
				g-value			*A*-value (10^{-4} cm^{-1})		
				g_0	$g_{//}$	$g_{\perp}$	A_0	$A_{//}$	$A_{\perp}$
VS	H_2O	3.0		1.969	1.935	1.986	106	186	66
VO(γ-pga1)		2.0	620 sh.†; 765	1.967	1.941	1.979	103	176	67
VO(γ-pga2)	H_2O	2.5		1.967	1.942	1.975	102	178	64
VO(γ-pga3)		3.0		1.965	1.941	1.978	102	177	65
		4.0	600 sh.†; 780	1.965	1.940	1.977	102	177	65
		5.0		1.966	1.942	1.977	102	176	65

Sh.† = absorption shoulder.

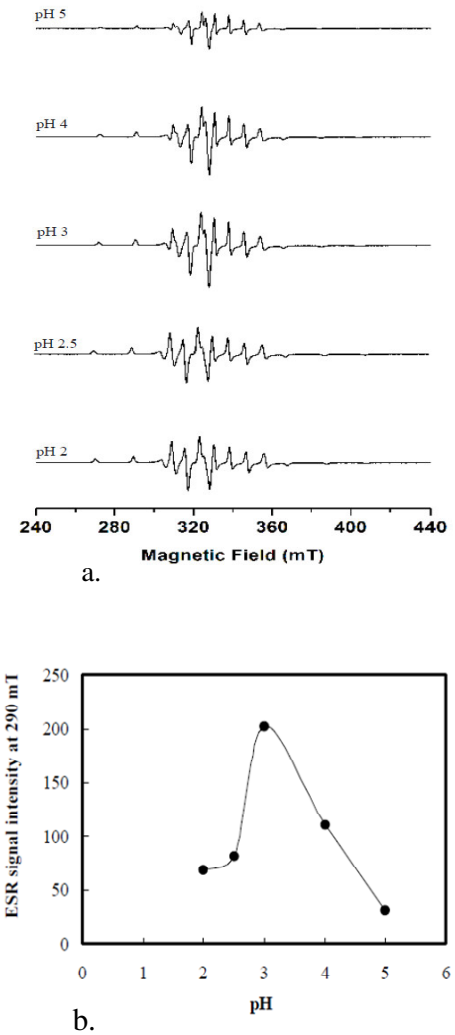

Figure 1. Typical ESR spectra of VO(γ-pga1) complex in aqueous solution as a function of pH at liquid nitrogen temperature (a) and the changes of ESR signal intensity at 290 mT of VO(γ-pga1) complex with pH (b).

and 177 × 10^{-4} cm^{-1} for [2 × carboxylate (2O)–VO–$(OH_2)_2$] are very close to the observed values of A_0 and $A_{//}$ for the complex as shown in Table 1.

Based on the above results, the equatorial coordination sphere of VO^{2+} in all three VO(γ-pga) complexes was proposed to be same as previously reported complex [2 × carboxylate (2O)–VO–$(OH_2)_2$] (Figure 2) (Karmaker and Saha, 2008).

Evaluation of *in vitro* insulin-mimetic activity of VO(γ-pga) complexes

To evaluate *in vitro* insulin-mimetic activity of VO(γ-pga) complexes, we performed an *in vitro* assay based on inhibition of free fatty acid (FFA) release from isolated rat adipocytes treated with epinephrine, which is a simple and convenient method compared to the use of radioisotope reagents (Nakai et al., 1995). With this assay, VO(γ-pga) complexes showed significantly ($p < 0.05$) better insulin-mimetic activity compared to VS. Moreover, all three VO(γ-pga) complexes exhibited similar effects in inhibiting of FFA release (Table 2) as observed in previously reported VO(γ-pga) complex in which the average molecular weight of γ-pga was 500 kDa (Karmaker et al., 2006).

Evaluation of *in vivo* anti-diabetic activities of VO(γ-pga) complexes

Following the *in vitro* experiments, we examined the blood glucose normalizing effects of vanadyl compounds following daily oral administrations of VS and three VO(γ-pga) complexes to STZ-mice. The changes of the blood glucose levels in saline-treated non-diabetic normal std: ddY mice and STZ-mice treated with saline, VS (doses: 10 mg V kg^{-1} body mass) and VO(γ-pga) com-plexes (doses: 10 mg V kg^{-1} body mass) for 28 days are shown

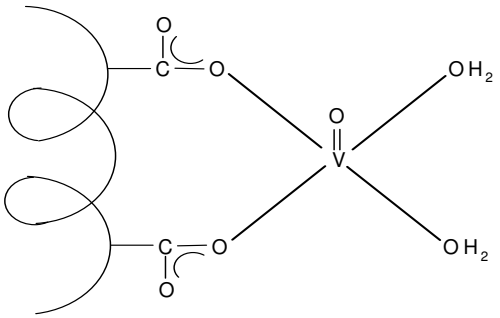

Figure 2. The structure of VO(γ-pga) complexes.

Table 2. FFA-releasing IC_{50} values of VS and three VO(γ-pga) complexes in isolated rat adipocytes treated with epinephrine.

Sample	FFA assay
	IC_{50} (mM)
VS	0.38 ± 0.15
VO(γ-pga1)	0.18 ± 0.05*
VO(γ-pga2)	0.21 ± 0.08*
VO(γ-pga3)	0.15 ± 0.03*
VO(γ-pga)	0.17 ± 0.05* (Karmaker et al., 2006)

Significance: *$p<0.05$ *vs.* VS.

in Figure 3. The γ-pga was not administrated in this study because it had no hypoglycemic activity in STZ-mice (Karmaker et al., 2006). The blood glucose level in the VS-treated STZ-mice was not significantly decreased compared to the saline-treated STZ-mice although the dose of VS is same as complex (Figure 3). The blood glucose level remained above 400 mg dL^{-1} (22.2 mM), which is significantly high compared to the saline-treated normal std: ddY and VO(γ-pga) complexes-treated STZ-mice, respectively. When VO(γ-pga1) (dose: 10 mg Vkg^{-1} body mass) and VO(γ-pga2) (dose: 10 mg V kg^{-1} body mass) complexes were administered, the elevated blood glucose levels (526 mg dL^{-1} or 29.2 mM) quickly decreased in 194–210 mg dL^{-1} (10.8 - 11.7 mM) after three days, which is very close to the blood glucose level (142 mg dL^{-1} or 8 mM) in the saline-treated non-diabetic normal std: ddY mice (Figure 3). However, the blood glucose levels increased again from sixth day and remained around 280 - 290 mg dL^{-1} (15.6 - 16.1 mM) until eleventh day. Then the blood glucose levels again gradually decreased and sustained around 180 – 200 mg dL^{-1} (10 - 11.1 mM) for the last 14 days although the administration doses of the complexes were same.

On the other hand, administration of the VO(γ-pga3) complex at a dose of 10 mg V kg^{-1} body mass resulted in a gradual decrease in the blood glucose level of STZ-mice and remained approximately 180 - 200 mg dL^{-1} (10 - 11.1 mM) for the last 14 days; this value is also close to the blood glucose level in the nondiabetic normal std: ddY mice (Figure 3). There is no significance difference between the blood glucose levels in all three VO(γ-pga) complexes-treated STZ-mice and the saline-treated non-diabetic normal std: ddY mice for the last 14 days, indicating that the elevated blood glucose level in STZ-mice is normalized by the oral administration of all three VO(γ-pga) complexes for 28 days. These results also strongly support our previous observation, where the elevated blood glucose level in diabetic mice was partially improved by the oral treatment with VO(γ-pga) complex for 16 days in which the average molecular weight of γ-PGA was 500 kDa (Karmaker et al., 2006).

The daily food intake in saline-treated STZ-mice was significantly high compared to that of the saline-treated normal std: ddY mice (data not shown) indicating the high urinary losses of glucose in STZ-mice. On the other hand, the daily food intake was decreased in VS- and VO(γ-pga) complexes-treated STZ-mice after 2 - 3 days and remained constant throughout the experiment. Moreover, there was no statistically significant difference in food consumption in the case of saline-treated non-diabetic normal std: ddY mice and the STZ-mice treated with three VO(γ-pga) complexes (data not shown), suggesting that the food intake was corrected due to the suppression of urinary losses of glucose (Karmaker et al., 2008). Similarly, water intake in VS- and in all three VO(γ-pga) complexes-treated mice was significantly reduced after two days compared to that of the saline-treated STZ-mice (data not shown). On the other hand, water intake in saline-treated non-diabetic normal std: ddY mice and in VO(γ-pga) complexes-treated STZ-mice was almost constant.

Daily changes in body mass in each group of mice are shown in Figure 4. There were no significant changes in body mass of the mice treated with VS and VO(γ-pga) complexes. However, the non-diabetic normal std: ddY mice gained body mass higher than STZ-mice as shown in Figure 4. As the administration of all three VO(γ-pga) complexes to STZ-mice result in lowering of blood glucose levels (Figure 3) without causing loss of body mass of the animals (Figure 4), it can be said that hyperglycemia is not modified by food uptake, but is normalized by the treatment with all three VO(γ-pga) complexes (Karmaker et al., 2008).

To determine the improvement of oral glucose tolerance ability in STZ-mice after the treatment with VS and all three VO(γ-pga) complexes for 28 days, an OGTT was performed. The blood glucose levels of the saline-treated STZ-mice increased to a maximal concentration of 505 mg dL^{-1} (28.1 mM) at 30 min after the oral administration of glucose (1 g kg^{-1} body mass) and then slightly decreased, being remained at a high level 341 mg dL^{-1} (18.9 mM) (Figure 5). In contrast, the elevation of the blood glucose levels in STZ-mice treated with VO(γ-pga) complexes increased to maximal concentration 265 - 301 mg dL^{-1} (14.7 - 16.7 mM) was significantly ($p<0.01$)

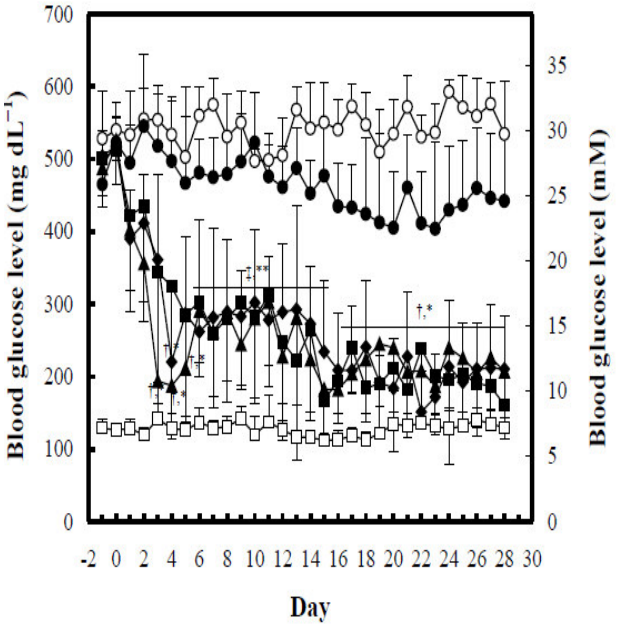

Figure 3. Changes of blood glucose levels in saline-treated non-diabetic normal std: ddY mice (□) and STZ-mice after daily oral administrations of saline (○), VS (●) and three VO-γ-PGA complexes (VO(γ-pga1) (▲); VO(γ-pga2) (♦); VO(γ-pga3) (■)) for 28 days (n=6). The dose of complex and VS was 10 mg Vkg^{-1} body mass. Complex and VS were prepared in saline solution (pH 3).
Significance: $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.05$ vs. saline-treated STZ-mice.
Significance: $^{*}p < 0.01$, $^{**}p < 0.05$ vs. VS-treated STZ-mice.

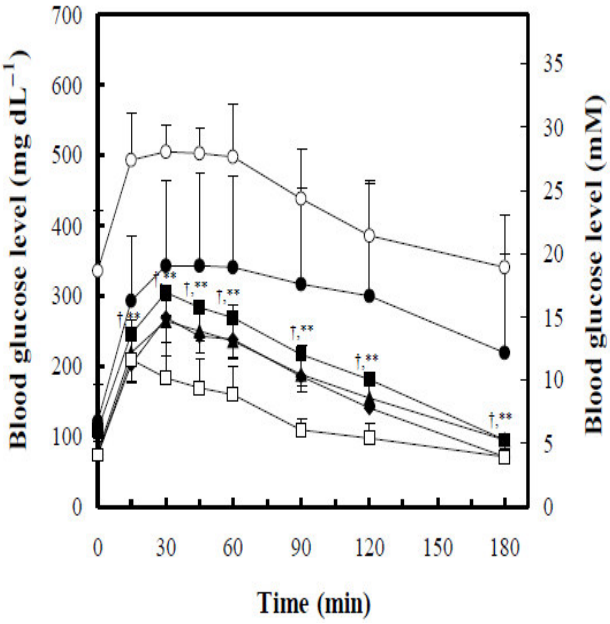

Figure 5. After 28 days treatment, the changes of blood glucose levels for oral glucose tolerance tests (OGTT) in saline-treated non-diabetic normal std: ddY mice (□) and STZ-mice after daily oral administrations of saline (○), VS (●) and three VO(γ-pga) complexes (VO(γ-pga1) (▲); VO(γ-pga2) (♦); VO(γ-pga3) (■) (n=6). 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.
Significance: $^{\dagger}p < 0.01$ vs. saline-treated STZ-mice.
Significance: $^{**}p < 0.05$ vs. VS-treated STZ-mice.

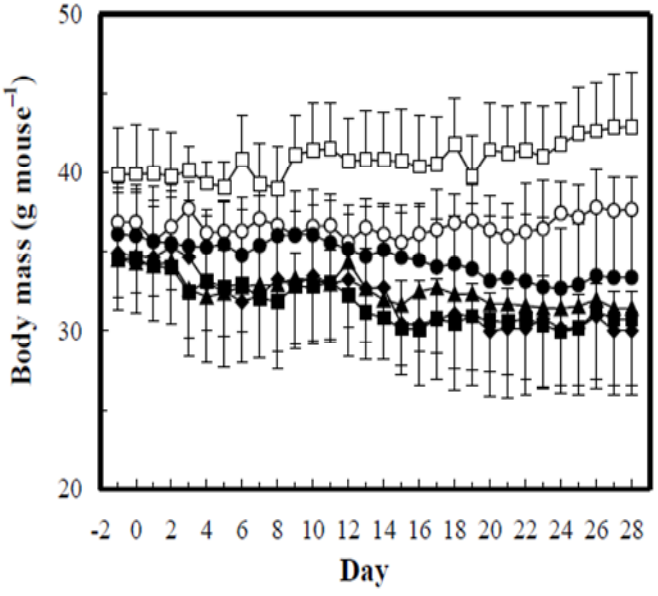

Figure 4. Changes of body mass in saline-treated non-diabetic normal std: ddY mice (□) and STZ-mice after daily oral administrations of saline (○), VS (●) and three VO(γ-pga) complexes (VO(γ-pga1) (▲); VO(γ-pga2) (♦); VO(γ-pga3) (■)) for 28 days (n = 6).

lowered than those of the saline and VS-treated STZ-mice, demonstrating that antidiabetic effect of the VO(γ-pga) complex was much better than that of VS. Moreover, the concentration profiles of the blood glucose levels in saline-treated non-diabetic normal std: ddY mice and all three VO(γ-pga) complexes-treated STZ-mice were comparable (Figure 5), although the maximal elevation of blood glucose level in nondiabetic normal mice was 209 mg dL^{-1} (11.6 mM) at 15 min after the administration of glucose. These results demonstrate that the impaired glucose tolerance in STZ-mice is ameliorated by the long time oral treatment with all three VO(γ-pga) complexes, as was observed in KKA^{y} mice by VO(γ-pga) complex treatment (Karmaker et al., 2007).

The concentration of HbA_{1c}, UN, GPT, GOT, TG, TCHO, FFA and insulin in the serum and systolic blood pressure of non-diabetic normal std: ddY mice and STZ-mice after the treatment with VS and three VO(γ-pga) complexes are summarized in Table 3. The HbA_{1c} concentration in VO(γ-pga) complexes-treated STZ-mice decreased significantly compared with those in saline- and VS-treated STZ-mice (Table 3). Similar results were observed when STZ-mice received orally VO(γ-pga) complex at doses of 5-10 mg (0.1 - 0.2 mmol) V kg^{-1}

Table 3. Improvements of HbA_{1C} levels, serum parameters, and blood pressure (BP) by treatments of VS, and three VO(γ-pga) complexes.

	std: ddY	STZ-mice				
	Saline	**Saline**	**VS**	**VO(γ-pga1)**	**VO(γ-pga2)**	**VO(γ-pga3)**
Dose (mg V kg^{-1})			**10**	**10**	**10**	**10**
n	**6**	**6**	**6**	**6**	**6**	**6**
HbA_{1C} (%)	3.3 ± 0.3	11.4 ± 0.8	9.4 ± 0.5	5.0 ± 0.9†,*	5.5 ± 0.7†,*	5.1 ± 0.6†,*
UN (mg dL^{-1})	29.6 ± 5.5	30.9 ± 4.3	26.2 ± 5.7	21.4 ± 3.4†	21.0 ± 4.6†	22.1 ± 2.1†
GOT (U L^{-1})	43 ± 12	71 ± 23	58 ± 15	46 ± 6	66 ± 12	57 ± 13
GPT (U L^{-1})	15 ± 0.7	36 ± 7	33 ± 9	30 ± 6	28 ± 6	29 ± 10
TG (mg dL^{-1})	132 ± 23	138 ± 16	135 ± 32	122 ± 7	116 ± 17	110 ± 22
TCHO (mg dL^{-1})	150 ± 33	204 ± 26	190 ± 44	139 ± 22†,*	139 ± 29†,*	133 ± 25†,*
FFA (mEq L^{-1})	1.469 ± 0.214	1.449 ± 0.074	1.477 ± 0.151	1.419 ± 0.159	1.559 ± 0.157	1.428± 0.202
Insulin (ng mL^{-1})	12.7 ± 2.7	4.4 ± 0.6	4.7 ± 0.7	5.9 ± 1.4	5.9 ± 0.9	6.0 ± 1.2
BP (mm of Hg)	110 ± 5	140 ± 8	126 ± 9	111 ± 6†	114 ± 5†	112 ± 7†

Significance: $^{\dagger}p<0.01$ *vs.* saline-treated STZ-mice.
Significance: $^{*}p<0.05$ *vs.* VS-treated STZ-mice.

body mass (HbA_{1c} = 6.9 ± 1.6%) and saline alone (HbA_{1c} = 10.0 ± 0.3%) for 16 days (Karmaker et al., 2006). These results indicate that all three VO(γ-pga) complexes treatment provide glycemic control in type 1 DM mice (Koenig et al., 1976). The serum UN concentration, which indicates the degree of renal disturbance, in VO(γ-pga) complexes-treated STZ-mice was significantly lowered compared with that of saline-treated STZ-mice. However, the UN level in VS-treated STZ-mice slightly lowered compared with that of saline-treated STZ-mice (Table 3). It has been reported that the kidney is one of the important target organs of vanadium toxicity (Hansen et al., 1982; Jandhyala and Hom 1983). Significant decrease in serum UN concentration in VO(γ-pga) complex-treated STZ-mice (Table 3) indicates normal kidney function and thus reveals the nontoxic nature of all three VO(γ-pga) complexes. The serum GOT and GPT concentrations, which show the degree of liver disturbance, were lowered in VO(γ-pga) complexes-treated STZ-mice compared with those in saline-treated STZ-mice. Serum TG and TCHO levels in STZ-mice following the treatment with VO(γ-pga) complexes were lowered significantly compared to those in saline- and VS-treated STZ-mice, indicating that cholesterol metabolism improved by the administration of all three VO(γ-pga) complexes. The serum FFA concentration in VO(γ-pga) complexes-treated STZ-mice was almost same as that in saline-treated STZ-mice. However, the serum insulin concentration in the previous groups was significantly higher than that in the later group (Table 3). The systolic blood pressure significantly ($p<0.01$) lowered upon the administration of VO(γ-pga) complexes comparatively with the saline-treated STZ-mice, indicating that all three VO(γ-pga) complexes have a low or no hepatic and renal toxicities and improve cholesterol metabolism, insulin level and blood pressure in STZ-mice.

The present results also show that all three VO(γ-pga) complexes have similar anti-diabetic activities on STZ-mice. Therefore, the total vanadium distribution in the STZ-mice after the treatment with VS and VO(γ-pga1) complex for 28 days was determined using inductively coupled plasma mass spectrometer (ICP-MS) (Table 4). Vanadium was detected in almost all tissues, particularly bones, liver, muscles, spleen, heart, kidneys, brain, lungs and pancreas, in this decreasing order, in STZ-mice treated with VS and VO(γ-pga1), but it was not detectable in the saline-treated STZ-mice and in normal std: ddY mice. Among the insulin-sensitive tissues, skeletal muscle accounts for the high uptake of vanadium. Interestingly, complex showed a higher distribution of vanadium in the skeletal muscle compared to VS (Table 4). These results suggest that complex has a potent hypoglycemic effect due to higher distribution of vanadium to skeletal muscle (Saha et al., 2006; Adachi et al., 2006), which is an important target tissue for insulin, than that of VS. This also supports the previous finding that vanadium compounds treat hyperglycemia in STZ-mice (Saha et al., 2006; Adachi et al., 2006). Recently, Kawabe et al. (2006) evaluated the mode of action for anti-diabetic activity of vanadium compounds, VS, bis(picolinato)oxo-vanadium(IV) complex, $VO(pa)_2$ and bis(maltolato)oxovanadium(IV) complex, $VO(ma)_2$, in isolated rat adipocytes using inhibitors for glucose and fatty acid metabolism. Oxovanadium(IV) ion and its complexes have been revealed to act on at least four sites involving insulin-dependent signal transduction system, glucose transporter and phosphodiesterase. Our study regarding the mechanism responsible for the anti-diabetic activity of VO(γ-pga) complexes is still under investigation and the results will be reported.

Conclusion

In conclusion, all three VO(γ-pga) complexes are orally

Table 4. Organ distribution of vanadium in STZ-mice treated with VS and VO(γ-pga1) complex.

Vanadium content (μg g^{-1} of wet tissue)									
Group	**Bone**	**Liver**	**Muscle**	**Spleen**	**Heart**	**Kidney**	**Brain**	**Lung**	**Pancreas**
VS	88.0 ± 7.8	33.0 ± 4.4	24.4 ± 1.8	5.6 ± 2.4	1.5 ± 0.1	1.1 ± 0.1	1.2 ± 0.3	1.0 ± 0.1	0.8 ± 0.2
VO(γ-pga1)	89.8 ± 4.9	35.3 ± 4.5	25.0 ± 1.0	5.3 ± 0.5	1.7 ± 0.3	1.3 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	0.9 ± 0.3

active vanadyl(IV)-biopolymer complexes in which γ-pga having average molecular weights 480–4700 kDa with a $VO(O_4)$ coordination environment. These complexes are useful for treating not only type 1 diabetic STZ-mice but also type 2 diabetic KKA^y mice (Karmaker et al., 2007). This study shows that the administration of VO(γ-pga) complexes for a longer period of time ameliorates the diabetic state in STZ-mice more efficaciously than our previous study (Karmaker et al., 2006).

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