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Synthesis, Structure, and Physiological Effects of Peroxovanadium(V) Complexes Containing Amino Acid Derivatives as Ancillary Ligands

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We have synthesized and structurally characterized novel peroxovanadium(V) compounds containing amino acid derivatives as ancillary ligands; *N*-(4-imidazolylmethyl)-L-alanate (imala) and *N*-(4-imidazolylmethyl)-L-phenylalanate (imphe). The structure of the imala complex was determined using X-ray crystallography. These compounds stimulated the proliferation of H4IIEC3 rat hepatoma cells at low doses but were cytotoxic at high doses. They also exhibited insulin-mimetic effects.

Vanadium forms a variety of peroxovanadium(V) complexes (pVs) of chemical interest. In addition, the physiological properties of these complexes have attracted much recent attention because they have been found to have antitumor and insulin-mimetic effects in higher animals.[1] The mechanisms underlying the pV insulin-mimetic effect have been previously investigated;[2] activation of the tyrosine kinase activity of the insulin receptor (IR) appears to be responsible for the effect.[3] Various ancillary ligands have been combined with pVs to create compounds with insulin-mimetic activity; however, the relationship between pV ligand structure and the physiologic properties of the pV compound is unclear. Furthermore it would be likely that non-peroxo vanadium complexes in the cell might react with reactive oxygen species such as peroxide. Thus, the study of peroxovanadium complexes in vitro will provide an insight into the metabolic pathways of vanadium complexes administrated in vivo.

Future development of pV compounds for use in clinical applications will require further investigation into the ligand property–activity relationship as it relates to the mechanism of the insulin-mimetic effects. Ancillary ligands commonly used in previous studies of stable pV compounds include 2,2′-bipyridine (bpy),[4] 1,10-phenanthroline (phen),[5] 2-picolinate (pic),[6] and 2,6-dipicolinate (dipic).[7] Typically, these pV compounds exhibit high cytotoxicity.[8] The high cytotoxicity, which may be related to the use of ligands that do not resemble biological substances, poses a significant barrier to the use of pV compounds in curative medicine.

In the present study, we attempted to decrease the cytotoxicity of pV compounds by replacing the above non-biological ancillary ligands with amino acid derivatives. These derivatives, which differ only in their side arms, were *N*-(4-imidazolylmethyl)-L-alanate (imala) and *N*-(4-imidazolylmethyl)-L-phenylalanate (imphe) (Figure 1). After synthesizing and structurally characterizing these pV compounds [pV(imala) and pV(imphe), respectively], we investigated their physiologic effects in H4IIEC3 rat hepatoma cells.

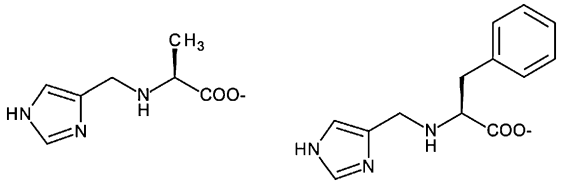

Figure 1. Structures of the amino acid derivative ligands imala (*N*-(4-imidazolylmethyl)-L-alanate; left) and imphe (*N*-(4-imidazolylmethyl)-L-phenylalanate; right).

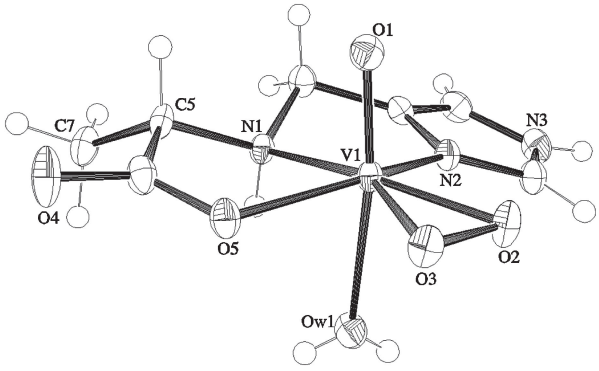

Figure 2. ORTEP drawing of pV(imala).

The pV(imala) and pV(imphe) compounds were prepared according to standard methods.[9] The structure of pV(imala) was determined using X-ray crystallography;[10] a molecular model of this compound is shown in Figure 2. The vanadium atom in this compound is seen to adopt a heptacoordinate, pentagonal-bipyramidal structure that is typical for a monoperoxovanadium(V) compound with a heteroligand. Although we were unable to obtain crystals of pV(imphe), its structure can be reasonably assumed to be similar to that of pV(imala), as the imphe and imala backbones are identical.

We used the MTT method to evaluate the cytotoxic activity of pV(imala) and pV(imphe) in H4IIEC3 rat hepatoma cells.[11]

Table 1. Results of the cytotoxic examination

Dose /μmol L^{-1}	Cell viability control/%	
	[$VO(O_2)$(imala)(H_2O)]·$2H_2O$	[$VO(O_2)$(imphe)]·$0.5H_2O$
3	108.09	106.53
10	112.43	112.54
30	111.14	104.14
100	104.73	93.64
200	92.16	28.38
300	93.75	2.09
500	10.71	0.81
800	1.23	0.93

Since the present peroxovanadium complexes are fairly stable at physiological pH at least in vitro, we believe that they have sufficient lifetimes to interact with some biological materials. As summarized in Table 1, low-dose treatment of the cells with pV(imala) or pV(imphe) stimulated cell proliferation, with the number of cells reaching its maximum value for both compounds at a concentration of 10 μM. Both compounds exhibited significant cytotoxicity at higher doses, although their cytotoxicity thresholds differed. Whereas pV(imphe) treatment at 200 μM reduced cell viability by approximately 28%, pV(imala) treatment at 200 μM only slightly reduced cell viability. No significant reduction of the cell viability (11%) was observed for pV(imala) until it reached a concentration of 500 μM. Notably, this large difference in cytotoxicity was caused by a small modification of the amino acid ligand: the substitution of a methyl group [in pV(imala)] for a phenyl group [in pV(imphe)]. The reason for this large difference is unclear at present, but lipophilicity is likely to be an important factor; the higher lipophilicity of the phenyl group would facilitate passage of pV(imphe) through the cell membrane.

The physiologic effects of some pV compounds have been investigated in other cell lines.[12–15] In RINm5F rat pancreatic cancer cells, $[VO(O_2)_2(1,10\text{-phen})]^-$ stimulates cell proliferation at low doses but is cytotoxic at higher doses, like pV(imala) and pV(imphe) in our case. Exposure of the RINm5F cells to 1 or 3 μM $[VO(O_2)_2(1,10\text{-phen})]^-$ for 48 h increased cell viability by approximately 15% and 20%, respectively.[14] On the other hand, treatment at 100 μM reduced cell viability by approximately 90%.[15] A similar phenomenon has also been observed in PC12 rat adrenal pheochromocytoma cells.[13]

At low doses, the effects of diperoxo(1,10-phenanthroline)oxovanadate(V) [bpV(phen)] on cell growth are similar to those of pV(imala) and pV(imphe).[13,14] However, the concentration at which bpV(phen) begins to inhibit cell viability is approximately 1/10 that of pV(imala) and pV(imphe). Thus, the latter compounds are much less cytotoxic than bpV(phen), possibly because their ligands are derived from biological substances. Thus, unlike bpV(phen), pV(imala), and pV(imphe) might be therapeutically effective for treatment of diabetes at a dose that is only weakly cytotoxic.

The effects of insulin on target cells are initiated by the binding of insulin to the insulin receptor (IR) on cell membranes.[16,17] This binding enhances the intrinsic protein tyrosine kinase activity of the IR β-subunit, leading to autophosphorylation of the β-subunit, the subsequent phosphorylation of several cytosolic IR substrates, and the activation of two key signaling pathways. One of these pathways exerts mitogenic and growth-promoting effects via phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). The second pathway, which involves phosphorylation of AKT (protein kinase B), affects glucose metabolism and protein synthesis. The multiple physiologic effects of insulin are thus mediated by ERK1/2 and AKT phosphorylation of various factors.

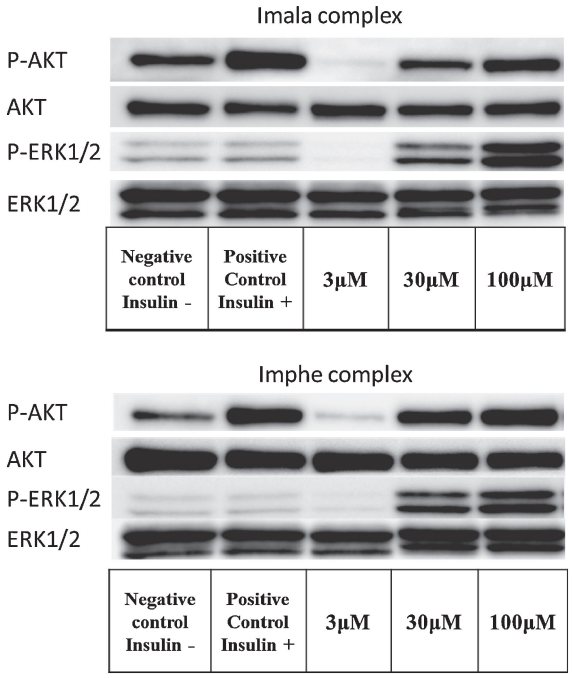

Figure 3. Effects of pV(imala) and pV(imphe) on insulin-related signaling. Cells were grown in 12-well plates until they reached 90–100% confluence, starved for 1 h, and exposed to pV(imala), pV(imphe), insulin (positive control), or vehicle (negative control). After 30 min, the cells were harvested in lysis buffer and sonicated. Equal amounts of total protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to poly(vinylidene difluoride) membranes for immunoblot analysis. Blots were visualized with GE ECL Plus western blotting detection reagent according to the manufacturer's instructions.

To further examine the possibility that low-cytotoxicity pV compounds might be used at low doses in the treatment of diabetes, we investigated the effects of pV(imala) and pV(imphe) on insulin-related intracellular signaling. Specifically, we examined the effects of these compounds on the phosphorylation of AKT and ERK1/2 in H4IIEC3 cells using western blotting (Figure 3). Cells treated with insulin or vehicle were used as positive and negative controls, respectively. As shown in Figure 3, exposure of the cells to 30 or 100 μM pV(imala) and pV(imphe) induced the phosphorylation of both AKT and ERK1/2 in a dose-dependent manner, demonstrating that both compounds activate insulin signaling pathways in H4IIEC3 cells.

It was considered that pV(imala) and pV(imphe) induced the phosphorylation of the insulin receptor which is the upstream

of AKT and ERK1/2. On the other hand it was reported that the vanadate inhibits the protein tyrosine phosphatase 1B (PTP1B).[18] We have clarified the dual effects of hydrogen peroxide (ROS) on the insulin signaling and also examined the vanadate effect on insulin signaling. It was found that the enhanced insulin signaling (pAKT activation and PTP1B inhibition) was observed when vanadate and low concentration of hydrogen peroxide were coadministered.[18] Since pV(imala) and pV(imphe) consist of pentavalent vanadium atom as in vanadate and peroxide, they are reasonably considered to exhibit similar effects to the above case. Therefore the insulin signaling was transmitted in the cells.

The phosphorylation-inducing effect of pV(imala) and pV(imphe) on the AKT substrate was weaker than that of insulin, whereas their phosphorylation-inducing effect on the ERK1/2 substrate was stronger than that of insulin. This observation indicates that pV compounds are more potent than insulin as promoters of cell differentiation and cell growth, thus raising the possibility that they also pose an increased risk of carcinogenesis relative to insulin. However, the extent of AKT and ERK1/2 phosphorylation induced by treatment of the cells with the pV compounds at 3 μM was less than that induced by insulin treatment, indicating that the insulin-mimetic effects of the pV compounds was dependent on their concentration.

In this study, we synthesized and structurally characterized novel pV compounds containing imala and imphe as ancillary ligands. An assessment of their physiologic effects in vitro demonstrated low toxicity and insulin-mimetic activity at concentrations less than 100 μM. Therefore, these compounds are expected to be useful in the treatment of diabetes at low doses.

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9 Imala and imphe were prepared by a reaction of alanine and phenylalanine, respectively, with 4-imidazolecarbaldehyde using $NaBH_4$ as a reductant. For synthesis of pV(imala), 2.0 mL of 4 M potassium hydroxide (2.0 mmol) was added to a suspension of vanadium pentoxide (0.18 g, 1.0 mmol) in 1 mL of water. The mixture was stirred while heating. After the resulting clear solution was cooled in an ice bath, Himala·2HCl·EtOH·1.5H_2O (0.63 g, 2.0 mmol) was added and the solution was stirred for 1 h. Then, 10% hydrogen peroxide (0.68 g, 2.0 mmol) was added slowly to the solution. The pH of the resulting red solution was adjusted to 4.3 by the addition of 1 M hydrochloric acid. Storage of this solution in a refrigerator for 2 days resulted in the deposition of red columnar crystals. Yield: 66% (0.21 g) Anal. Calcd for $[VO(O_2)(imala)(H_2O)]\cdot 2H_2O = C_7H_{16}N_3O_8V$: C, 26.18; H, 5.03; N, 13.09%. Found: C, 25.64; H, 4.84; N, 12.68%. The pV(imphe) compound was prepared by a similar method. Yield: 39% Anal. Calcd for $[VO(O_2)(imphe)]\cdot 0.5H_2O = C_{13}H_{15}N_3O_{5.5}V$: C, 44.33; H, 4.29; N, 11.93%. Found: C, 44.49; H, 4.45; N, 12.04%.

10 X-ray structure data: Rigaku AFC7R diffractometer, ω scans, Mo Kα radiation ($\lambda = 0.71070$ Å), graphite monochrometer, $T = 200(2)$ K, structure solution with direct method (SHELX97: G. M. Sheldrick, *Program for Crystal Structure Solution from Diffraction Data*, University of Göttingen, Göttingen, Germany, **1997**), refinement against F^2 for all non-hydrogen atoms. Data collection: crystal dimension $0.50 \times 0.10 \times 0.10\,mm^3$, monoclinic, space group $C2$ (No. 5), $a = 24.001(3)$, $b = 6.351(2)$, $c = 8.449(3)$ Å, $\beta = 100.78(2)°$, $V = 1265.1(6)$ Å^3, $Z = 4$, $D_{calcd} = 1.686\,g\,cm^{-3}$, $\mu = 0.825\,mm^{-1}$, $F(000) = 664$, data collected 1609, unique data 1572 ($R_{int} = 0.012$), 199 refine parameters, GOF = 1.077, final R indices ($I > 2\sigma(I)$) ($R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, $wR_2 = [\Sigma w(F_o^2 - F_c^2)/\Sigma w(F_o^2)]1/2$): $R_1 = 0.0248$, $wR_2 = 0.0664$. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre (no. CCDC-855902). Copies of the data can be obtained free of charge on application to CCDC, 12, Union Road, Cambridge, CB2 1EZ, U.K. (fax: +44 1223 336033); e-mail: deposit@ccdc.cam.ac.uk.

11 Cells were grown in 96-well plates. When the cells reached 90–100% confluence, the cells were place in a starvation state for 24 h. Then the cells were treated with or without vanadium complexes for 48 h. MTT assay was carried out according to kit description. Then the absorbance was measured at 595 nm by a microplate reader. The cell viability was calculated by normalizing the absorbance to the corresponding control.

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