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A New Insulin-mimetic Vanadyl Complex, (*N*-Pyridylmethylaspartate)oxovanadium(IV) with $VO(N_2O_2)$ Coordination Mode, and Evaluation of its Effect on Uptake of D-Glucose by Ehrlich Ascites Tumour Cells

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Abstract

Because it has been confirmed that the vanadyl(IV) ion and its complexes act as insulin mimetics, a new organic vanadyl complex, (*N*-pyridylmethylaspartate)oxovanadium (VOPASP) with $VO(N_2O_2)$ coordination mode, was prepared. Development of a simple and rapid in-vitro assay is needed for recognition of potent insulin-mimetic complexes.

Treatment of Ehrlich ascites tumour cells with 2-deoxyglucose in the presence of vanadyl sulphate, or other vanadyl complexes with the same coordination mode (VOPASP, bis(picolinate)oxovanadium (VOPA) and bis(6-methyl picolinate)oxovanadium (VOMPA)), in the presence of 2-deoxy-D-[1-^{3}H]glucose ([^{3}H]deoxyglucose), resulted in concentration-dependent uptake of 2-deoxyglucose by the cells. The responses of the cells to the vanadyl complexes were reflected, in part, by results obtained from the free fatty acid-releasing assay using rat adipocytes.

These results show that the in-vitro assay with Ehrlich ascites tumour cells provides an accurate and rapid assessment of glucose uptake by the cells. The assay is proposed as a means of predicting the insulin-mimetic activity of the vanadyl complexes and for studying the mechanism of action of the complexes.

Diabetes mellitus is a disease associated with absolute or relative insulin deficiency. Therapy for insulin-dependent diabetes mellitus, which is characterized by absolute insulin deficiency and involves many serious secondary complications such as diabetes retinopathy, diabetic nephropathy and diabetic neuropathy, requires the daily subcutaneous injection of insulin (Atkinson & MacLaren 1990). Recently, administration of vanadate ion (+5 oxidation state of vanadium) to rats with streptozotocin-induced diabetes was found to normalize their blood glucose levels (Heyliger et al 1985; Mayerovitch et al 1987). Later, it was reported that vanadyl (+4 oxidation state of vanadium) complexes are effective after oral administration to rats with streptozotocin-induced diabetes (Sakurai et al 1990a; McNeill et al 1992).

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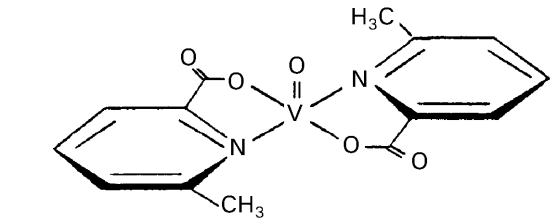
These findings stimulated research on insulin-mimetic vanadium complexes (Duckworth et al 1988; Jackson et al 1988; Lazaro et al 1989; Blondel et al 1990; Cam et al 1993; Orvig et al 1995; Sakurai & Tsuji 1997). In the course of attempting to develop new insulin-mimetic vanadium complexes it was found that vanadyl complexes are less toxic than those of the vanadate ion and are present exclusively in the active form in rat cells and tissues (Sakurai et al 1990b; Nakai et al 1995).

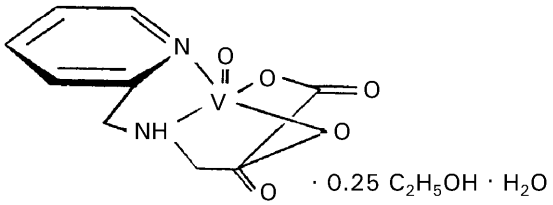
Several observations indicate that a highly active glucose transport system is common to neoplastic and to transformed animal cells (Warburg 1956; Salter & Weber 1979). Ehrlich ascites tumour cells, which depend primarily on glycolysis for the provision of energy, have a highly efficient glucose transport system dependent upon glucose transporters (GLUT) GLUT1 and GLUT3 for the maintenance of growth (Crane et al 1957; Chan et al 1983; Au et al 1997). These cells can be easily

propagated and used as an experimental system without the need for a complicated procedure to separate cells (Cuppoletti et al 1981), such as the free fatty acid-releasing assay, which might injure cellular membranes and also needs a number of rats (animals) for isolation of adipocytes (Nakai et al 1995). Therefore, Ehrlich ascites tumour cells have been used to measure glucose transport activity (Cuppoletti et al 1981; Yamasaki et al 1992).

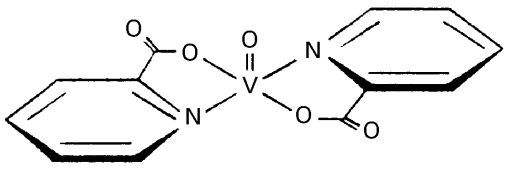
Vanadyl and vanadate ions have been reported to activate glucose transporters by enhancing their intrinsic activity in peripheral adipocytes (Lerea et al 1989; Strout et al 1990; Okumura & Shimazu 1992). Because of this action, in this study an in-vitro assay using Ehrlich ascites tumour cells was investigated to determine whether (*N*-pyridylmethylaspartate)oxovanadium(IV) (VOPASP), a newly synthesized vanadyl complex with $VO(N_2O_2)$ coordination mode designed to be orally absorbed, has insulin-like action, in terms of glucose uptake, similar to that of other vanadyl complexes with same coordination mode—bis(picolinate)oxovanadium(IV) (VOPA) (Sakurai et al 1995) and bis(6-methylpicolinate)oxovanadium (VOMPA) (Fujimoto et al 1997) (Figure 1). This is the first study in which these cells have been used to evaluate the insulin-mimetic activity of vanadyl complexes.



Bis(6-methylpicolinate)oxovanadium
(VOMPA)



(N-pyridylmethylaspartate)oxovanadium
(VOPASP)



Bis(picolinate)oxovanadium
(VOPA)

Figure 1. Structures of vanadyl complexes with $VO(N_2O_2)$-coordination mode.

Materials and Methods

Vanadyl complexes

The tetradentate ligand *N*-(2-pyridylmethyl)-L-aspartate was prepared according to the method described by Meiske et al (1980) and isolated as the lithium salt. VOPASP was synthesized by adding solid $BaCl_2$ (5 mmol) to a solution (10 mL) of $VOSO_4$ (5 mmol) with stirring. The precipitate of $BaSO_4$ was removed by filtration and a solution (10 mL) of Li_2(L-*N*-(2-pyridylmethyl)-L-aspartate) (5 mmol) was added to the filtrate. The resulting dark blue solution was stirred for 30 min and the precipitate was collected. The complex was purified by recrystallization from aqueous ethanol. VO(L-*N*-(2-pyridylmethyl)-L-aspartate) (0·25 C_2H_5OH)(H_2O): C, 37·45; H, 4·05; N, 8·33; found: C, 37·73; H, 4·24; N, 8·31. $\nu_{V=O}$: 967 cm^{-1} (KBr disk). VOPA and VOMPA were prepared as previously reported (Sakurai et al 1995; Fujimoto et al 1997). The partition coefficients (P) of vanadyl complexes were estimated by use of a 1-octanol-saline system (Table 1).

Uptake of 2-deoxy-D-glucose by Ehrlich ascites tumour cells

Four-week-old male ddy mice, 15–25 g, were inoculated intraperitoneally with 0·2 mL saline containing 10^6 Ehrlich ascites tumour cells harvested from 7–10-day-old tumours. Tumour cells were harvested by centrifugation and washed twice with phosphate-buffered saline to remove blood cells. The final cell suspension was prepared in

Table 1. Effect of vanadyl sulphate and vanadyl complexes on release of free fatty acids from adipocytes and on uptake of 2-deoxyglucose by Ehrlich ascites tumour cells, and the partition coefficients of the vanadium compounds between 1-octanol and saline (1 : 1, %w/w).

Compound	Inhibition of free fatty acid release IC50 (mM)	Incorporation of 2-deoxyglucose (% of insulin)	Partition coefficient
Vanadyl sulphate	6·00	26·9 ± 9·4	0·033
VOPA	0·47	36·5 ± 3·0	0·330
VOMPA	0·45	21·9 ± 3·0	0·595
VOPASP	0·25	71·6 ± 2·6	0·086

Values are means ± standard deviation of results from three independent experiments; the concentration of the vanadium compound was 1 mM.

saline. The cell count was determined with a haemocytometer. Samples (20 μL) of the cell suspension (5×10^7 cells mL^{-1}) were placed in an Eppendorf tube containing 50 μL insulin (Sigma, St Louis, MO), vanadyl sulphate or vanadyl complex, 130 μM 2-deoxyglucose (Nakalai Tesque, Kyoto), and 150 μL Krebs-Ringer Hepes (KRH) buffer, pH 7·4, and equilibrated at 37°C in a shaking water bath for 5 min. Reaction was initiated by adding 1·5 μL 2-deoxy-D-[1-^{3}H]glucose ([^{3}H]2-deoxyglucose, 444 GBq mmol^{-1}, 37 mBq mL^{-1}; Amersham Life Science, Tokyo, Japan), and the mixture was further incubated. The reaction was terminated by filtering the solution through a glass fibre filter coated with a layer of Xtalscint solid scintillation medium (2·5 cm, Beckman Instruments Japan, Tokyo), after which the solution was rapidly washed with KRH buffer (3×5 mL). The radioactivity remaining on the filter was determined by liquid-scintillation counting (Beckman Instruments).

Free fatty acid-releasing assay

Male Wistar rats, 200 g, were killed by decapitation under ether anaesthesia and the adipocytes were isolated from the epididymal fat pads by a method reported elsewhere (Nakai et al 1995; Sakurai et al 1995). Fat tissue was chopped with scissors and digested for 1 h at 37°C in Krebs-Ringer bicarbonate (KRB) buffer, pH 7·4, containing 20 mg bovine serum albumin (BSA, essentially fatty acid free, Sigma) and 2 mg mL^{-1} collagenase (Wako Chemicals, Osaka). Adipocytes were then separated from the undigested tissues by filtration through nylon mesh (250 μm), washed three times with KRB buffer without collagenase, and adjusted to $2{\cdot}5 \times 10^6$ cells mL^{-1}. Isolated adipocytes in siliconized vials were pre-incubated at 37°C for 0·5 h with different concentrations of vanadyl complexes in 1 mL KRB buffer containing 20 mg mL^{-1} BSA in the absence of glucose. 10^{-5} M adrenaline (Sigma) was then added to the reaction mixtures and the resulting solutions were incubated at 37°C for 3 h. The reactions were stopped by cooling in ice water, and the mixtures were centrifuged at 1200 rev min^{-1} for 10 min. Free fatty acid levels in the supernatant were determined with a NEFA kit (Wako Chemicals).

Statistical analysis

The statistical significance of differences was tested by use of Student's two-tailed *t*-test for paired data; $P < 0{\cdot}05$ was considered to be indicative of significance.

Results and Discussion

It has been suggested that vanadate compounds activate GLUT4, an isoform of GLUT, in rat adipocytes and thereby improve insulin sensitivity by enhanced receptor binding (Dubyak & Kleinzeller 1980; Shechter & Karlish 1980; al-Attas et al 1995). Because the glucose transport mechanism of Ehrlich ascites tumour cells involves GLUT1 and GLUT3 (Au et al 1997) the purpose of this study was to determine whether vanadyl ion and its complexes also enhance glucose uptake in these cells. The time-course of 2-deoxyglucose transport into Ehrlich ascites tumour cells was measured by use of the tracer [^{3}H]2-deoxyglucose as the non-metabolizable analogue. In the presence of 1 mM vanadyl sulphate or VOPA, the rate of 2-deoxyglucose uptake increased gradually up to 20 min as shown in Figure 2; the onset of action in the presence of the same concentration of insulin was more rapid, occurring within 10 min. This difference between the rates of incorporation might be associated with the rate of diffusion of vanadyl complexes into the cells (Olefsky 1978). On the other hand, insulin directly accelerates glucose uptake by increasing the number of available transport units and thus enhancing the maximum transport capacity (Minkel & Petering 1978; Cong et al 1997; Goodyear & Kahn 1998).

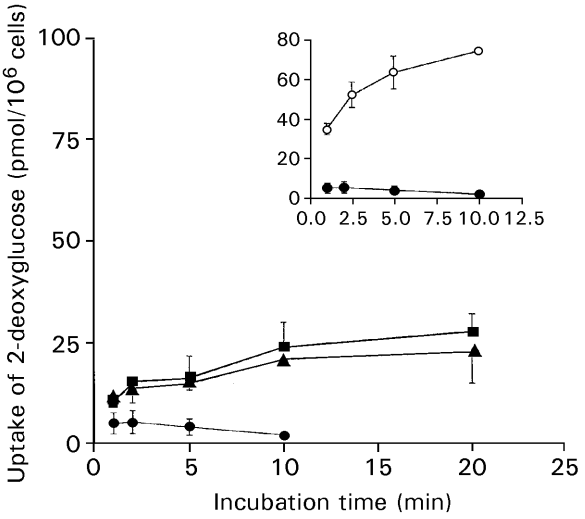


Figure 2. Time-dependent uptake of 2-deoxyglucose by Ehrlich ascites tumour cells. The cells (10^6) were incubated with 1 mM of the test compounds (●, control; ○, insulin (inset), ▲, vanadyl sulphate; ■, VOPA (bis(picolinate)oxovanadium)) in the presence of 2-deoxyglucose (130 μM), [^{3}H]2-deoxyglucose (55·5 kBq/1·5 μL), and KRH buffer (pH 7·4, 150 μL) at 37°C. Cells were collected by centrifugation at the indicated time intervals, washed rapidly with KRH buffer (3×5 mL) and their radioactivity was determined. Each value, expressed as pmol 2-deoxyglucose/10^6 cells, is the mean ± standard deviation of results from at least three independent experiments.

The effect of the concentration of vanadyl sulphate and vanadyl complexes on 2-deoxyglucose uptake in Ehrlich ascites tumour cells was examined with an incubation time of 20 min. The fraction of 2-deoxyglucose taken up by Ehrlich ascites tumour cells depended on the concentration of the vanadyl complexes examined; incorporation of 2-deoxy- glucose was increased to 1·9 and 16 times that of the basal uptake level by 1 mM VOPA and VOPASP, respectively (Table 2). Vanadyl sulphate and VOMPA had essentially no effect. Although the partition coefficient of VOPASP (Table 1) suggested that it would have less effect on 2-deoxyglucose uptake by Ehrlich ascites tumour cells and would be less effective at inhibiting free fatty acid release from adipocytes, VOPASP was highly effective at activating 2-deoxyglucose uptake and inhibiting release of free fatty acids compared with vanadyl sulphate, VOPA, and VOMPA (Table 1).

In insulin-stimulated glycogen synthesis and enhancement of glucose incorporation into cells, a tyrosine kinase pathway associated with the binding of phosphatidylinositide 3-kinase to tyrosyl phosphorylated insulin receptor substrate-1 plays a central role in the regulated movement of GLUT from intracellular vesicles to the cell surface (Oatey et al 1997; Clark et al 1998). The vanadate ion, which is a potent phosphotyrosine phosphatase inhibitor, promotes phosphorylation of the insulin receptor (Tamura et al 1984; Marshall & Monzon 1987; Okumura & Shimazu 1992). On the basis of the results presented in Table 2, 2-deoxyglucose uptake by Ehrlich ascites tumour cells is assumed to be principally based on the regulation through true influx rather than net uptake accompanied by significant efflux (Chandramouli & Carter 1977). It has been reported, however, that glycogen synthesis stimulated by vanadate and vanadyl ions is independent of the tyrosine phosphorylation of the insulin receptor, although it is associated with increased tyrosine phosphorylation of insulin receptor substrate 1 by activation of protein tyrosine kinases (Pandey et al 1998). Therefore, the concentration-dependent 2-deoxy- glucose uptake into Ehrlich ascites tumour cells elicited by VOPASP probably occurs as a result of changes in the intrinsic activity of GLUT1 in the cells (Okumura & Shimazu 1992; Van Epps-Fung et al 1997). It has also been reported that vanadate ion stimulates 2-deoxyglucose uptake even after depletion of the insulin receptor from the cell surface and also stimulates glucose transport in the absence of ATP (Green 1986). Therefore, the contributions of VOPA and VOPASP to overall 2-deoxyglucose transport in Ehrlich ascites tumour cells might be the result of changes at the post-receptor level that are effective at a level distal to the insulin receptor. It is possible that vanadyl complexes act by increasing tyrosyl phosphorylation of a non-insulin-receptor protein which is required to stimulate GLUT1-mediated glucose transport in the Ehrlich cells, because signalling molecules might lie in the signalling pathway to glucose transport (Tamura et al 1984; Van Epps-Fung et al 1997). To confirm this possibility, it is necessary to measure the state of phosphorylation of GLUT1 in Ehrlich ascites tumour cells in the presence of vanadyl ion and its complexes.

In adipocytes, fatty acid synthesis from pyruvate is increased when glucose transport in cells is increased by addition of insulin (Haystead & Hardie 1986). Insulin also suppresses the decomposition of fatty acids in fat tissues and this is accompanied by reduction of cAMP levels (Lehninger et al 1993). Therefore, measurement of free fatty acid release from adipocytes could yet provide means of assessing the effect of vanadyl complexes on glucose uptake in Ehrlich ascites tumour cells. Previous work in our laboratory demonstrated that vanadyl sulphate, VOPA, VOMPA, and bis(pyrrolidine-*N*-carbodithioate)oxovanadium (VOPS) enhanced incorporation of glucose by rat adipocytes, and suppressed release of free fatty acids from adipocytes stimulated with adrenaline (Watanabe et al 1994; Sakurai et al 1995; Fujimoto

Table 2. Effects of insulin, vanadyl sulphate, and vanadyl complexes on uptake of 2-deoxyglucose by Ehrlich ascites tumour cells.

Compound	Concn (mM)	Uptake of 2-deoxyglucose (pmol/10^6 cells)
Saline	–	15·0 ± 6·1
Insulin	0·1	56·1 ± 13·0**
Insulin	1·0	75·1 ± 5·2**
Vanadyl sulphate	0·1	17·5 ± 1·8
Vanadyl sulphate	1·0	20·4 ± 7·3
VOPA	0·1	14·9 ± 0·2
VOPA	0·5	17·5 ± 1·0
VOPA	1·0	27·6 ± 2·5*
VOPASP	0·1	24·0 ± 1·8*
VOPASP	0·5	47·0 ± 4·1**
VOPASP	1·0	53·9 ± 2·2**
VOMPA	0·5	12·3 ± 1·2
VOMPA	0·7	13·8 ± 0·7
VOMPA	1·0	16·6 ± 2·5

The cells (10^6) were incubated with the test compounds in the presence of 2-deoxyglucose (130 μM), [^{3}H]2-deoxyglucose (55·5 kBq/1·5 μL), and KRH buffer (pH 7·4, 150 μL) at 37°C. Cells were collected by centrifugation, washed rapidly with KRH buffer 3 × 5 mL) and their radioactivity was determined. Each value is the mean ± standard deviation of results from at least three independent experiments. VOPASP = (*N*-pyridylmethylaspartate)oxovanadium, VOPA = bis(picolinate)oxovanadium, VOMPA = bis(6-methyl picolinate)oxovanadium. *$P < 0·05$, **$P < 0·01$ compared with result from saline.

et al 1997). In the current study, when adipocytes were pre-incubated with different concentrations of vanadyl sulphate and VOPASP in the absence of glucose and then incubated with adrenaline, VOPASP also dose-dependently suppressed free fatty acid release. Table 1 summarizes the 50% inhibition concentration for free fatty acid release (IC50). The results agree well, in part, with those from measurement of 2-deoxyglucose uptake by the in-vitro assay using Ehrlich ascites tumour cells, as presented in Table 2.

In conclusion, this study indicates that the vanadyl complexes examined here have structure-dependent effects on the insulin-sensitive glucose transmembrane transport process in Ehrlich ascites tumour cells. An in-vitro system using these cells would thus provide an accurate assessment of glucose uptake and an effective means of predicting the insulin-mimetic effects of vanadyl complexes. By use of the proposed in-vitro evaluation system it has been found that VOPASP is a potent complex in the treatment of insulin-dependent diabetes mellitus. However, it remains to be determined whether vanadyl complexes act on GLUT1 directly or indirectly via some other factors regulating glucose transport.

It has recently been reported that when glucose transport into rat adipocytes was stimulated by noradrenaline a significant increase of GLUT4 was not detected in the plasma membrane, whereas insulin directly stimulated the rate of glucose transport by increasing the level of the cell-surface GLUT4 (Shimizu et al 1996, 1998). This implies that the mechanism of noradrenaline-stimulated glucose transport into adipocytes is not because of translocation of GLUT4 but probably because of an increase in the intrinsic activity of GLUT4 mediated by a cyclic AMP-dependent pathway. We are continuing our study to confirm whether vanadyl complexes regulate the translocation of GLUT 1 during the process of glucose uptake into Ehrlich ascites tumour cells.

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