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Tyrosine phosphorylation and morphological transformation induced by four vanadium compounds on MC3T3E1 cells

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Abstract

The present study was performed to determine the phosphotyrosine-protein levels induced by insulin and by four vanadium derivatives in MC3T3E1 osteoblast-like cells. We have also attempted to associate these patterns with the vanadium-induced growth and morphological changes of such cells. Vanadate (Vi), vanadyl (VO), bis(maltolato)oxovanadium (IV) (BMOV) and bis(maltolato)dioxovanadium (V) (BMV) stimulate cell growth in a narrow range of concentration, but are also inhibitors for the cells at high concentrations. Vanadium-treated cells displayed clear changes in their morphology after overnight incubation. However, BMV was the least cytotoxic and the weakest inducer of morphological changes. All the compounds promote the phosphorylation of tyrosine residues in several proteins. This effect was more pronounced at low than at high doses. At low doses (10 μM), BMV showed a phosphorylation pattern similar to that of insulin, while Vi, VO and BMOV induced strong phosphorylation of cell proteins. The present findings suggest that the vanadium-induced growth regulation and morphological changes in MC3T3E1 osteoblast-like cells are associated with the ability of these agents to increase the phosphotyrosine protein levels and to inhibit phosphotyrosine phosphatases. These properties are dependent on the oxidation state as well as on the organic ligand which coordinates the vanadium atom. (Mol Cell Biochem **198**: 119–128, 1999)

Key words: vanadate, vanadyl, maltol complexes, proliferation, cytotoxicity, tyrosin phosphorylation, phosphotyrosine phosphatases, osteoblast-like cells

Introduction

Numerous studies have demonstrated the insulin-mimetic actions of vanadium compounds in different cell types [1–3]. Evidence also suggests their possible role as growth factors, acting on mitogenesis, cell differentiation and morphological transformation. It has been shown that vanadate stimulates DNA-, collagen-, and extracellular matrix protein-synthesis in cloned cell lines as well as in primary cultures of both rat calvaria cells and chondrocytes [4–7].

We have previously shown that vanadate increased cell proliferation, protein content and glucose consumption in two osteoblast-like cell lines (MC3T3E1 and UMR106) [8–10]. In addition, we have demonstrated that some vanadium derivatives such as orthovanadate, vanadyl and pervanadate induced morphological changes on the fibroblastic cell line Swiss 3T3, an effect which correlated with the biphasic cell proliferation pattern. At low doses (10 μM) these vanadium compounds caused a stimulatory effect upon fibroblasts proliferation, together with slight morphological changes. On the other hand, at higher concentrations, pervanadate, vanadate and to a lesser extent vanadyl, strongly inhibited cell growth with dramatic alterations on cell morphology. The changes observed were dependent on both the oxidation state and the coordination geometry of the compound investigated [11].

As we have previously shown [8], 10^{-7} M insulin stimulated osteoblast-like cell proliferation and inhibited cell differentiation as assessed by alkaline phosphatase activity. It is not yet clear how vanadium derivatives exert their biological effects at cellular level. Although vanadium compounds and

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insulin induce similar actions, growing evidence now supports the hypothesis that they may affect different elements in the insulin-signal transduction pathways [12]. The earliest events in insulin action are the autophosphorylation and stimulation of tyrosine kinase activity of β-subunit insulin receptor, induced by insulin binding to the α-subunit [13]. Subsequently, insulin receptor tyrosine kinase phosphorylates endogenous substrates such as IRS-1 (insulin receptor substrate-1), that has been the most widely characterised. After that a cascade of phosphorylation takes place dowstream; MAPkinases and $p90^{rsk}$ become activated, which in turn will induce different events. Insulin also stimulates $p70^{s6k}$ protein by an independent pathway. Most evidence suggest that vanadate does not stimulate either the autophosphorylation or endogenous tyrosine phosphorylation activity of insulin receptor kinase, although this point remains controversial [14]. There is also plenty of information pointing to vanadium compounds as specific inhibitors of phosphotyrosine phosphatases (PTPases) [15]. Thus, by preventing dephosphorylation, these compounds are able to increase the phosphotyrosyl content of key-protein molecules in the insulin signalling cascade, resulting in the induction of the MAPkinases as well as $p70^{s6k}$ activating pathway [16, 17]. These effects may arise from an inhibitory action of vanadate upon PTPases [18]. On the other hand, aqueous peroxovanadium species (pV) inhibit PTPases and subsequently lead to the activation of the insulin receptor tyrosine kinase (IRK). Some pV compounds show greater potency on the insulin receptor phosphorylation than on any other tyrosine kinase receptors such as the epidermal growth factor receptor. This suggests a relative specificity of pV compounds on the insulin receptor [19].

It is well established that protein phosphorylation and dephosphorylation are critical components involved in the regulation of various cellular activities including proliferation, transformation and differentiation [20]. Tyrosine phosphorylation of key regulatory proteins is considered to be a major event in the growth factor-mediated signal transduction pathways [21]. Many studies show that vanadium derivatives induce tyrosine phosphorylation of several proteins [22]. Wang *et al*. [23] demonstrated that vanadate increased the tyrosine phosphorylation of 55 and 64 kDa proteins in CSV3-1 cells (SV40-transformed 3T3 T cells). Vanadate also caused a selective induction of the c-jun and junB expression [23]. The fact that both insulin and vanadate may act by different pathways is clinically important, since it is related to the possibility of avoiding defects associated with insulin resistance.

Several reports have shown the ability of various vanadium derivatives to promote transformations on different cell lines [3, 24, 25]. The mechanism by which vanadate induces morphological changes is not currently known, even though the inhibition of PTPases has been suggested [20].

The aim of our study was to determine the phosphotyrosine protein pattern induced by insulin as well as by four vanadium derivatives: orthovanadate, vanadyl, bis(maltolato)oxovanadium (IV) (BMOV), and bis(maltolato)dioxovanadium (V) (BMV). We have also attempted to establish the association between the tyrosine phosphorylation pattern induced by these compounds and their ability both to regulate cell growth and to cause morphological changes on the osteoblast-like MC3T3E1 cells.

Materials and methods

Materials

Sodium orthovanadate (Vi) and maltol (3-hydroxy-2-methyl-4-pyrone) were purchased from Sigma (St. Louis, MO, USA) and human insulin from Beta (Argentina). Vanadium (IV) oxide sulfate (vanadyl sulfate) was provided by Merck (Darmstadt, Germany), tissue culture material by Corning (Princeton, NJ, USA), Dulbecco's Modified Eagles Medium (DMEM) and trypsin-EDTA by Gibco (Gaithersburg, MD, USA) and fetal bovine serum (FBS) from Gen (Argentina). All other chemicals used were of analytical grade from Sigma.

Bis(maltolato)oxovanadium (IV) (BMOV) was prepared as previously described by McNeill [26]. Aqueous solutions of maltol and vanadyl sulfate were mixed in a 2:1 ratio. The pH of the solution was raised to 8.5 and the solution was heated with reflux overnight; the green compound was collected upon cooling. The solid product was characterized by infrared spectroscopy. Its magnetic moment was 1.76 BM indicating one unpaired electron. Bis(maltolato)dioxovanadium (V) (BMV) was obtained as described by Elvingson et al. [27]. Briefly, 10 mM vanadate and 20 mM maltol in water were mixed (1:2 molar ratio) and the pH of the solution was kept between 6.5 and 7.0. The solution was protected from light and was used within 48–72 h. The UV-visible spectrum of maltol showed two strong absorption bands in the UV region (214 and 274 nm). Based on the band at 274 nm we estimated the concentration of uncomplexed maltol in the reaction mixture. The value obtained 6.6% was in good agreement with the one reported by Elvingson *et al*. under the same experimental conditions.

The biotin-enhancer 4G10 Western blot kit for anti phosphotyrosine was from Upstate Biotechnology Incorporated (UBI) (Lake Placid, NY, USA). Kit: biotin conjugate anti phosphotyrosine monoclonal antibody, clone 4G10 [28], streptavidin-alkaline phosphatase. Substrate solution: 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) plus nitroblue tetrazolium (NBT).

Solutions

Fresh stock solutions of vanadyl (IV) sulphate (VO), sodium ovanadate (Vi), BMV and BMOV were prepared in distilled

water at 10 or 100 mM concentration as we previously described [10].

Cell culture

Osteoblastic MC3T3E1 cells were grown in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FBS at 37°C, 5% CO_2. When 70–80% confluence was reached, cells were subcultured using 0.1% trypsin - 1 mM EDTA in Ca^{2+}–Mg^{2+} free phosphate buffered saline (PBS) [8–10]. For experiments, cells were grown in six-well plates at a density of $2.5.10^4$ cells/well. When cells reached 70% confluence, the monolayers were washed twice with DMEM. Cells were incubated overnight either with insulin or the vanadium compounds at different doses in serum free DMEM. We previously tested that the free ligand (maltol) does not affect cell proliferation or morphology.

Whole-cell homogenate and cell fraction preparation

MC3T3E1 osteoblast-like cells were grown to confluency in 150 cm^2 flasks. Cells were washed with PBS, resuspended in a homogenizing buffer (25 mM Tris, 250 mM sucrose, 2 mM EDTA, 5 mM β-mercaptoethanol, 1 mM PMSF, pH 7.4), and sonicated three times at 60 psi for 15 sec. The homogenate was centrifuged at 1,000 × g for 10 min, and the supernatant was further centrifuged at 10,000 × g / 4°C for 30 min. Membranes were then isolated from the supernatant by centrifugation at 105,000 × g for 60 min at 4°C. The resulting pellet was solubilized in 0.1% Triton X-100 / PBS. Protein concentration in both, supernatant and membrane fractions was determined by the method of Bradford [29]. This material was stored at –20°C until the PTPase activity determination.

PTPases activity measurement

This activity was estimated by the potency of cell extract (50 µg protein/ml) to hydrolyze *p*-nitrophenyl phosphate (pNPP) according to Li *et al*. [30]. The assay (1 ml) consisted of 5 mM pNPP in 50 mM Hepes, pH 7.5, the enzyme source and different concentrations (0–100 µM) of vanadium compounds. After 30 min of incubation period, the optical density was measured at 405 nm.

Cell proliferation assay

A mitogenic bioassay was carried out as described by Okajima *et al.* [31] with some modifications. Briefly, cells in twenty-four-well plates were washed with PBS and fixed with 5% glutaraldehyde/PBS at room temperature for 10 min. Then, they were stained with 0.5% crystal violet/25% methanol for 10 min. After that, the dye solution was discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted using 0.5 ml/well 0.1 M glycine/HCl buffer, pH 3.0/30% methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample dilution. We have previously shown that under these conditions, the colorimetric bioassay strongly correlated with cell proliferation measured by cell counting in Neubauer chamber ($y = 0.938 \times - 0.127$, $r^2 = 0.97$, $p < 0.001$) [8, 9].

Morphological assay

Cells growing into six well-plates were incubated overnight either with insulin or different vanadium compounds in serum-free DMEM or media alone (control or basal condition). After two washes with PBS, the monolayer was fixed with methanol for 5 min at room temperature and stained with 1:10 dilution of Giemsa for 10 min. Finally, the plates were washed with water and the morphological changes induced by vanadium compounds and insulin were observed by light microscopy.

Detection of tyrosine phosphorylated proteins by Western blot

After overnight incubation with insulin or vanadium derivatives, cells were washed with cold PBS pH 7.4, scraped in the sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue, 10% glycerol) and boiled for 5 min. Then 50 µl of the extract (corresponding to 100 µg protein) were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels (SDS-PAGE) as described [32]. In each experiment a sample corresponding to basal condition was included. Transfer procedures were performed overnight at 70 mA, and after protein transfer onto nitrocellulose, immunological determinations were performed with the UBI kit, following the provided protocols with slight modifications. Filters were blocked by incubation with 3% non-fat milk in PBS (pH 7.4) for 30 min at room temperature. The antibody binding was performed overnight at 4°C in PBS with 1 % bovine serum albumin (BSA), dilution 1:1000. Filters were washed twice with distilled water and incubated for 1 h at 37°C with streptavidin-alkaline phosphatase. After washing twice with distilled water, nitrocellulose was treated for 3–5 min with PBS-0.05 % Tween 20 and then washed four times with distilled water. Visualisation was performed by incubating the filters with alkaline phosphatase substrates NBT-BCIP until the colour developed.

Results

Effect of insulin and vanadium compounds on osteoblast-like cell proliferation

Figure 1 shows the cell proliferation assay, as measured by the crystal violet bioassay, after 24-h-culture of MC3T3E1 osteoblast-like cells with different concentrations of insulin (Fig. 1A) or vanadium derivatives (Fig. 1B). As can be seen in Fig. 1A, insulin stimulates cell proliferation in a dose response manner, with a maximal effect at 10^{-7} M. Comparably, vanadium compounds exert a biphasic effect (Fig. 1B). Low levels of vanadium slightly stimulate cell growth; the values were statistically significant at 2.5 μM for Vi ($p < 0.002$), VO ($p < 0.05$), and BMOV ($p < 0.05$), and between 2.5 ($p < 0.05$) to 10 μM ($p < 0.01$) for BMV. Over such concentrations, an inhibition of cell progression was observed with ED_{50} of 10, 20, and 22 μM for Vi, VO and BMOV respectively. According to this criteria, BMV was the least cytotoxic compound that induces a significant inhibition of cell proliferation at 100 μM dose ($p < 0.002$). Thus, we chose 10 μM concentration to further investigate the morphological changes induced by vanadium compounds. Under this dose, clear differences between the various vanadium compounds could be observed.

Induction of changes in cell morphology by vanadium compounds

The ability of different vanadium compounds to induce morphological changes was investigated in the MC3T3E1 osteoblast-like cells. After overnight incubation in a serum-free medium with or without vanadium derivatives, cells were fixed, stained and observed by light microscopy. Under these starved conditions, a moderate number of cells started to detach from the culture dishes. The monolayer of control and insulin-treated cells showed the aspect of a typical fibroblast-like culture (Fig. 2A and B). Cells were stellate in shape and exhibited slender lamellar expansions. These processes appeared to connect each other among neighbour cells. The nuclei contained moderately thick chromatin granules, while the cytoplasm showed numerous inclusions and vacuoles.

In general, vanadium-treated cultures displayed clear changes in their morphology. Cells became fusiform, with denser cytoplasm. Most processes were lost, the cell border appeared well-defined and the nuclei showed no major changes (Fig. 2C–F). Studies with different vanadium compounds showed that BMV was the weakest inductor of cell changes. Very slight changes were exhibited in MC3T3E1 osteoblast-like cells treated with 10 μM BMV (Fig. 2C) when compared to the control cells. Conversely, the same doses of other vanadium compounds induced pronounced changes with the

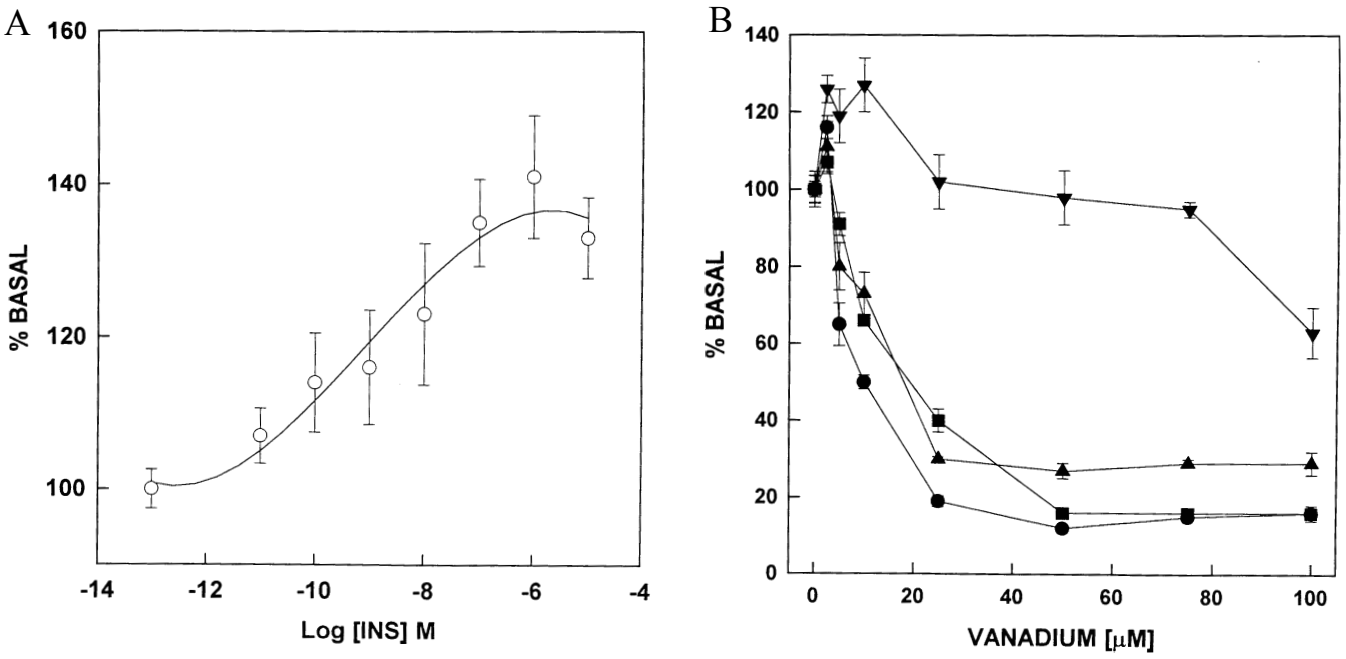

Fig. 1. Effect of insulin (A) and vanadium compounds (B) on MC3T3E1 osteoblast-like cell proliferation. Cells were incubated in serum-free DMEM (basal) alone or with different concentrations of insulin (○) , Vi (●), vanadyl (■), BMOV (▲), or BMV (▼), at 37°C for 24 h. Results are expressed as % basal, X ± S.E.M., n = 6. Basal values are $1.2.10^4$ cell/ml.

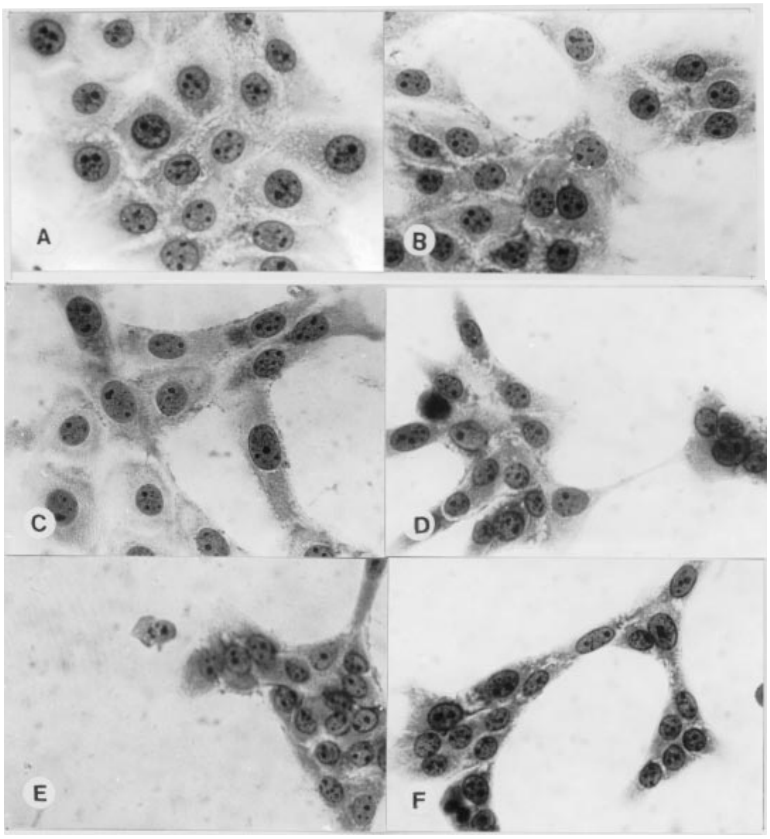

Fig. 2. MC3T3E1 osteoblast-like cells incubated in DMEM (control) (A); 10^{-7} M insulin (B), 10 μM BMV (C), VO (D), BMOV (E), Vi (F) at 37°C for 24 h. After this period, cells were stained with Giemsa. Obj. x40.

following order of potency: vanadyl < BMOV ≅ vanadate (Fig. 2D–F). These derivatives induced marked morphological changes, whose degree was dependent on the concentration of the vanadium derivative. A gradual loss of connections as well as an increase in the cytoplasm condensation were observed from 10–50 μM dose, while the survival of the cell culture was variable (picture not shown). At a concentration of 50 μM, the most pronounced changes were found and an important number of cells died and detached from the monolayer.

The comparison of the results obtained with the classical forms of vanadium (V) and (IV) (vanadate and vanadyl, respectively) with those found with their organic ligand complexes seems to indicate that the proliferative and transforming behaviour depends on the oxidation state as well as on the coordination geometry of vanadium.

Vanadium compounds and insulin effects upon protein tyrosine phosphorylation.

After overnight incubation in the presence of 10^{-7} M insulin, the phosphotyrosine pattern of MC3T3E1 cells was assessed by Western blot. Figure 3A shows that insulin induced tyrosine phosphorylation of a number of proteins over the basal control culture. Bands with molecular weight corresponding to 116, 85, 70, 60, 55, 50, 44 and 42 kDa were observed, whereas a band with 90–92 kDa was occasionally found, a fact that could be coincident with the β-subunit insulin-receptor.

We also evaluated the effect of four different vanadium derivatives with various oxidation states and coordination ligands. Overnight incubation of the cells with vanadium compounds in serum-free medium at different doses induced the tyrosine phosphorylation of several proteins. At a 10 μM dose, vanadium derivatives increased the phosphorylation of proteins with molecular masses corresponding to 130, 116, 92, 85, 76, 70, 60, 56, 50, 44, 42, 30 and 24 kDa (Fig. 3A). It was always observed that vanadate, vanadyl and BMOV appeared to be more potent than BMV to induce tyrosine phosphorylation in MC3T3E1 cells. The latter showed a response which resembled the insulin-stimulated phosphotyrosine pattern mainly in the range of 38–45 kDa bands.

At 25 μM, the vanadium-induced protein tyrosine-phosphorylation was weaker than that observed at 10 μM

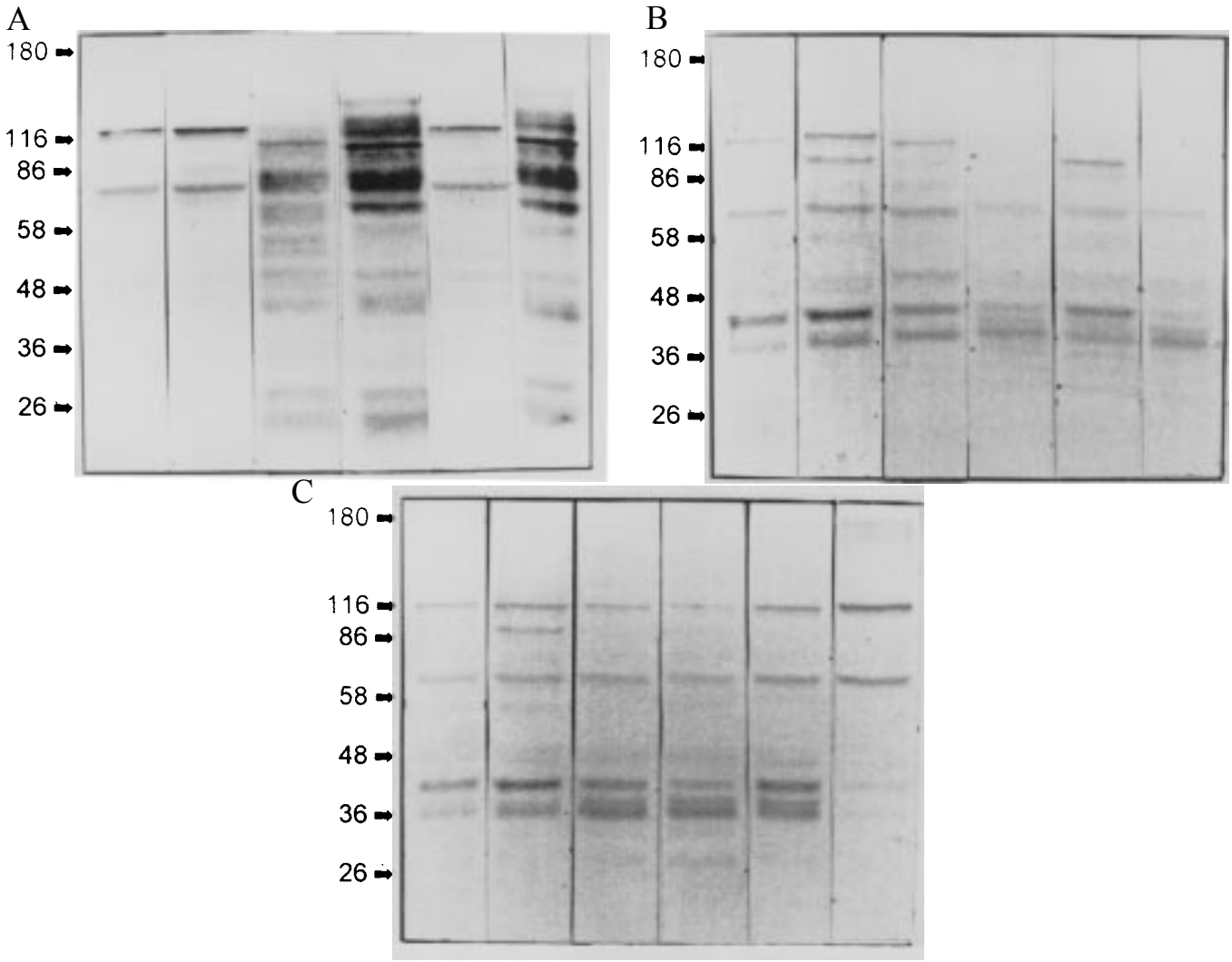

Fig. 3. Effect of vanadium compounds on phosphotyrosine protein pattern of MC3T3E1 cells. Cells were incubated with DMEM (lane 1) plus 10^{-7} M insulin (2) or Vi (3), VO (4), BMV (5), BMOV (6) at 10 μM (panel A), 25 μM (B), or 50 μM (C) for 24 h. After this period, proteins were separated by electrophoresis and analysed by Western blot using a monoclonal anti-phosphotyrosine antibody as described in Material and methods. Each panel is representantive of 3–5 independent experiments.

doses (Fig. 3B). Vanadate stimulated the phosphorylation of 116, 92, 85, 70, 60, 50, 44 and 42 kDa bands: this pattern was similar to that of insulin. The VO and BMOV exerted similar but weaker effects than vanadate, mainly by stimulating low molecular weight proteins (44, 42 and 36 kDa). On the other hand, BMV showed an effect which was intermediate between Vi and the other vanadium compounds.

When MC3T3E1 cells were incubated with 50 μM doses of VO, Vi, BMOV and BMV vanadium derivatives, a weak effect on tyrosine phosphorylation similar to that with insulin (Fig. 3C) was observed. The main bands phosphorylated in response to these vanadium derivatives were of 42, 36 and 30 kDa. On the contrary, BMOV stimulated the phosphorylation of 116 and 70 kDa proteins.

Vanadium sensitive PTPase in osteoblast-like cells

We tried to identify the PTPases sensitive to vanadium compounds in extracts of MC3T3E1 cells. Thus, osteoblastic extracts were separated in supernatant and membrane fractions. In a first series of experiments we tested the ability of BMOV and BMV to inhibit the activity of PTPases. Figures 4A and B show that both compounds inhibit the hydrolysis of pNPP mediated by PTPases from cytosolic (supernatant) and membrane fractions in a dose-dependent manner. However, the percentage of vanadium-sensitive PTPases accounted for about 50–60% of total activity. The ED_{50} of the vanadium sensitive PTPases was between 5–10 μM for both BMOV and BMV. Thus, the potency of these

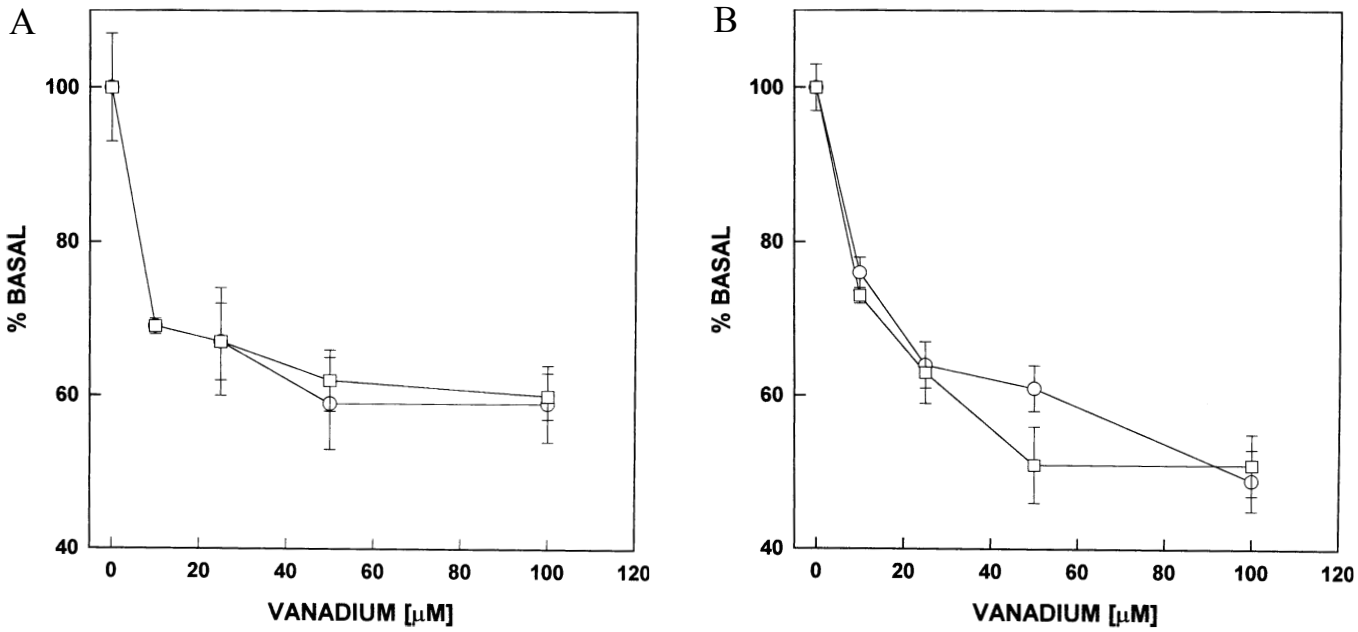

Fig 4. Effect of vanadium compounds on PTPase activity fractions. Supernatant (A) and membrane (B) fractions were incubated with pNPP in 50 mM Hepes buffer pH 7.5 with the indicated concentration of BMOV (□) or BMV (○). Basal activity was obtained in absence of inhibitors and corresponds to 11 and to 1.8 nmol pNP / mg protein . min (cytosolic and membrane fraction, respectively). Data represent mean ± S.E.M. of n = 9.

vanadium complexes to inhibit the activity of PTPases in MC3T3E1 fractions was also similar to the one found for vanadate (data not shown).

These results suggest that vanadium sensitive PTPases in osteoblast-like cells are located in cytosolic and membrane fractions, their activity being approximately 50% of the total cellular PTPase activity.

Discussion

The insulin-mimetic effects of both vanadate and vanadyl have been largely studied by *in vivo* and *in vitro* systems [1–3]. Although vanadate is absorbed from the gastrointestinal tract into blood, the effective dose is close to the toxic level [33]. Vanadyl is less toxic than vanadate but is poorly absorbed at physiological pH [26]. Many laboratories have been involved in the development of easily absorbed forms of vanadium with low toxicity to be administered in low doses.

The use of organic ligands for vanadium was promoted in order to obtain greater lipophilicity, which will improve the intestinal absorption. One of such ligands was the food additive maltol, which has a low toxicity. Complexes with vanadium (V) and vanadium (IV) have been developed and their stabilities and chemical structures determined. Orvig's group [34] has carried out studies to examine the stability of the BMOV in aqueous solution under different conditions. Kinetic results suggest that, under its administration, the prevalent species absorbed is the tetravalent vanadium. On the other hand, results obtained by Pettersson's group [27] demonstrated that at a 10/20 mM V/Maltol molar ratio, pH 6.5 and 0.150 M NaCl, the predominant vanadium species formed was $[VMa_2]^-$. Under these conditions, more than 90% of maltol was complexed according to ESR and ^{51}V NMR quantitative studies.

The effects of vanadium derivatives in bone-related cells are of great interest. Vanadium produces significant biological effects on bone development. On absorption, its compounds are distributed among several tissues and retained specially in bones [35]. On the other hand, it has been previously reported that vanadium deficiency causes growth inhibition and skeletal deformations [36].

In this study, we have tested the effect of vanadate, vanadyl and their complexes with maltol on certain growth parameters, using the model of the MC3T3E1 osteoblast-like cells in culture. We have compared these results with those obtained with insulin. In this cell line, insulin induced a dose-dependent proliferative effect (Fig. 1A). Vanadium compounds exert a slight mitogenic effect at low doses (Fig. 1B). This behaviour was also observed in other cell lines, like UMR106 and Swiss 3T3 [8, 11]. However, a strong inhibition of cell proliferation was observed over 10 μM dose with Vi, VO and BMOV. On the other hand, BMV showed the least cytotoxic effect on MC3T3E1 cells according to this assay.

In order to understand the effects of vanadium compounds on bone related cells, we have also assessed the morphological changes induced by vanadium in MC3T3E1 cells. Vanadium compounds produced changes in the culture from a fibroblast-like shape to spindle-like phenotype. The stronger effects were caused by vanadate, BMOV and vanadyl, while BMV produced weaker modifications (Fig. 2C).

We have previously shown that these four vanadium compounds regulate the growth of the UMR106 osteosarcoma cells [10], where vanadium derivatives exert a stimulatory effect between 20–50 μM. In addition, in this culture system no marked cytotoxicity was observed at higher doses of vanadium, in contrast to our present findings for MC3T3E1 cells. These results seem to indicate that MC3T3E1 cells are more sensitive to vanadium than UMR106 osteoblast-like cells.

It has been suggested that one of the possible mechanisms of action for vanadium derivatives is the regulation of phosphotyrosine protein level [18]. We have studied the tyrosine phosphorylation pattern induced by the four vanadium derivatives in MC3T3E1 cells. All the compounds promoted the phosphorylation of tyrosine residues in several proteins as insulin did (Fig. 3). The effect was more pronounced at low doses (10 μM) than at higher doses. The weak effect of the higher doses upon the phosphorylation pattern may be related to the cytotoxicity caused by the vanadium compounds (Vi, VO and BMOV) at these concentrations (Fig. 1B). At low doses, BMV showed a phosphorylation pattern similar to that of insulin (Fig. 3A), while vanadate, BMOV and vanadyl induced a strong phosphorylation of cell proteins.

A number of observations support the notion that protein tyrosine phosphorylation levels are important in the process of cell transformation [24]. Tyrosine kinase activities have been demonstrated to be associated with growth factor receptors. Thus, perturbations in the normal signalling pathway of growth factors could induce the cell transformations. Karlund [24] has suggested that this could occur by increasing the phosphorylation of one or more critical proteins. In this context, vanadate has been proposed to induce morphological changes by generating a highly refractive morphology, although high doses (1 mM / 6–12 h) were toxic and caused cell death. Moreover, Karlund showed that the transformed morphology was reverted 24 h after removing the vanadate from the media. He also showed that the transformed state was associated with high levels of phosphotyrosine proteins.

In a series of preliminary studies we have found that when media containing vanadium was replaced by vanadium-free - 10% serum DMEM, the osteoblast-like cells reverted to a normal morphology. The time required for reversion depended on the vanadium compound tested: 10 μM BMV pretreated cells reverted after 2 days of incubation with 10% serum-DMEM, while the same concentration of vanadate pretreated cells required 4 days, thus suggesting that BMV is the weakest transformer of this cell line.

Present observations on cell alterations (Fig. 2) agree with the above information and coincide with our current studies on cell proliferation (Fig. 1B) and the tyrosine phosphorylation pattern (Fig. 3). Thus, the stronger inductors of morphological changes (Vi and BMOV) promoted a higher level of tyrosine phosphorylation in these cells. Accordingly, BMV had the weakest effect both enhancing cell proliferation and inducing a phosphorylation pattern similar to that of insulin.

On the other hand, Sabbioni *et al*. [25] evaluated the toxicity of vanadium compounds by means of both the relative colony-forming efficiency and the induction of morphological transformations in the BALB/3T3 cells. They found that vanadyl cation was less cytotoxic than vanadate according to these parameters. These results agree with our observations since vanadate was stronger than vanadyl (IV) cation to cause morphological changes in the osteoblast-like cells (Fig. 2). However, BMV, a complex of vanadium (V) was the weakest cytotoxic compound, stimulating cell proliferation in a major range of concentration (Fig. 1B). It has been suggested that the ability of vanadium compounds to regulate cell growth and phosphotyrosine phosphorylation is associated with their action on the inhibition of specific PTPases. It could be argued that BMV could not inhibit PTPases, and then no enhancement of tyrosine phosphorylation would be observed. However, as we have previously reported [10], intestinal alkaline phosphatase is strongly inhibited by the four vanadium compounds with ED_{50} between 15–30 μM. It is therefore possible that BMV could be an inhibitor of cell PTPases. In this work we show that BMOV and BMV are both inhibitors of PTPase activity in MC3T3E1 fractions with nearly similar potency. These results seem to indicate that BMV is an effective inhibitor of PTPase in osteoblast-like cells. Thus, the weaker bioactivity of this vanadium derivative on the MC3T3E1 cells is not clear at present. We can hypothesized that the event involved in the BMV action may occur by a different phosphorylation pathway compared with other vanadium compounds. Alternatively, cells would metabolized this compound in a different way turning it less active. McNeill also found that BMV was less effective than BMOV and consequently required higher doses to lower the plasma glucose using animal models of diabetes [37].

In conclusion, the present study has demonstrated that vanadium compounds influence cell proliferation and morphological changes in MC3T3E1 osteoblast-like cells. These effects are, at least, partially associated with the ability of vanadium derivatives to enhance the phosphotyrosine protein levels by inhibition of PTPases. In addition, their bioactivity occurs in a narrow range of concentrations, over which cytotoxic effects are observed. These properties are not only dependent on the oxidation state but also on the organic ligand

coordinating the vanadium atom. Thus, the development of vanadium compounds for future treatment in human diseases should take in account these properties, as well as their possible transforming and cytotoxic effects. The side effect of oral administration of vanadium compounds has been evaluated in rodents and in less extention in human beings, being diarrhea and the decrease of fluid and food intake the major toxic effects [37]. Also it was shown that vanadium accumulates specifically in bone [36]. More work need to be done in order to establish the *in vivo* toxicity of these compounds.

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