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Impairment of mineralization by metavanadate and decavanadate solutions in a fish bone-derived cell line

Daniel M. Tiago · Vincent Laizé · M. Leonor Cancela · Manuel Aureliano

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Abstract Vanadium, a trace metal known to accumulate in bone and to mimic insulin, has been shown to regulate mammalian bone formation using *in vitro* and *in vivo* systems. In the present work, short- and long-term effects of metavanadate (containing monomeric, dimeric, tetrameric and pentameric vanadate species) and decavanadate (containing decameric vanadate species) solutions on the mineralization of a fish bone-derived cell line (VSa13) were studied and compared to that of insulin. After 2 h of incubation with vanadate (10 μM in monomeric vanadate), metavanadate exhibited higher accumulation rates than decavanadate (6.85±0.40 versus 3.95± 0.10 μg V/g of protein, respectively) in fish VSa13 cells and was also shown to be less toxic when applied for short periods. In longer treatments with both metavanadate and decavanadate solutions, similar effects were promoted: stimulation of cell proliferation and strong impairment (75%) of extracellular matrix (ECM) mineralization. The effect of both vanadate solutions (5 μM in monomeric vanadate), on ECM mineralization was increased in the presence of insulin (10 nM). It is concluded that chronic treatment with both vanadate solutions stimulated fish VSa13 cells proliferation and prevented ECM mineralization. Newly developed VSa13 fish cells appeared to be appropriate in the characterization of vanadate effects on vertebrate bone formation, representing a good alternative to mammalian systems.

Keywords Bone-derived cell line · Vanadate · Decavanadate · Insulin-mimetic properties · Vertebrate bone formation · Teleost fish *Sparus aurata*

Abbreviations

DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
MAPK	Mitogen-activated protein kinase
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PBS	Phosphate-buffered saline
PI3-K	Phosphatidyl inositol-3 kinase

Daniel M. Tiago and Vincent Laizé1 contributed equally to this work.

D. M. Tiago · V. Laizé · M. L. Cancela · M. Aureliano
Centre of Marine Sciences (CCMAR),
University of Algarve, Campus Gambelas,
8005-139 Faro, Portugal

M. Aureliano (✉)
Faculdade de Ciências e Tecnologia (FCT),
Universidade do Algarve, Campus de Gambelas,
8005-139 Faro, Portugal
e-mail: maalves@ualg.pt

Introduction

Vanadium, an essential trace element found in animals and higher plants (Anke 2004) and described to be accumulated in mammalian bone (Etcheverry et al. 1984), has been shown to display important biological effects in association with its physicochemical properties, e.g. insulin-like activity and regulation of phosphatases and ATPases activity (Nielsen and Uthus 1990). Vanadium, which easily transitions between three oxidation states, namely, V(III), V(IV) and V(V), has been proposed to be potentially important for its role as a biometal (Rehder 1999, 2003). Vanadate (V(V)), which is the most stable oxidation state in aerobic conditions, forms in solution monomeric vanadate species with different states of protonation, such as HVO_4^{2-} or $H_2VO_4^-$, depending on pH value. However, at physiological pH values and at millimolar range of concentrations, several *n*-meric species ($n=1$ to 10), normally ascribed as vanadate oligomers, can occur simultaneously in equilibrium, such as monomeric (V_1), dimeric (V_2) and tetrameric (V_4) vanadate species (Amado et al. 1993). Eventually, even decameric (V_{10}) vanadate species can be formed upon medium acidification (Aureliano and Gândara 2005). These vanadate oligomers species have been shown to interact with several proteins, thus affecting many biological systems (Crans et al. 2004). For instance, monomeric vanadate as been described to behave as a phosphate analogue, inhibiting (or, in some cases, stimulating) several phosphatases (acid, alkaline and protein phosphatases), phosphorylases, ribonucleases and ATPases (Crans et al. 2004). Conversely, other vanadate oligomers, such as the decameric vanadate, has been described to inhibit non-competitively several enzymes, among others, proteins involved in muscle contraction and regulation such as myosin ATPase and sarcoplasmic reticulum calcium ATPase, thus contributing for the several biological processes influenced by vanadate (Aureliano and Madeira 1994; Tiago et al. 2004; Aureliano and Gândara 2005).

The most important clinical effect of vanadium is probably its ability to mimic or to enhance the effect of insulin, as demonstrated in various *in vivo* and *in vitro* studies using streptozocin-treated diabetic rats (Heyliger et al. 1985; Meyerovitch et al. 1987) and adipocyte cultures (Dubyak and Kleinzeller 1980; Shechter and Karlish 1980). Insulin, a peptide that binds to specific membrane tyrosine kinase receptors, initiates intracellular signalling that either results in the regulation of protein, lipid, carbohydrate and mineral metabolism, or cell differentiation and proliferation, depending on the involved pathways: phosphatidyl inositol-3 kinase (PI3-K) pathway for metabolic effects or mitogen-activated protein kinase (MAPK) pathway for cell proliferation and differentiation effects (Cheatham and Kahn 1995; Cohick and Clemmons 1993; LeRoith 2000). Vanadium insulin-mimetic properties have been associated to the activity of tyrosine phosphatases and consequent activation of tyrosine kinase receptors, including the insulin receptor (Goldwaser et al. 2000; Shechter et al. 1995; Shisheva and Shechter 1993). In vertebrates, a major process regulated by insulin (and insulin-like growth factor 1) is linear bone growth (McCarthy et al. 2000; Moriyama et al. 2000). Vanadate was also shown to exhibit strong metabolic and mitogenic effects, as demonstrated by *in vitro* studies using mammalian bone-derived cell lines (and also using rat calvaria and chondrocyte primary cultures) (Canalis 1985a; Kato et al. 1987; Lau et al. 1988). These effects have been extensively studied using two mammalian cell lines, MC3T3-E1 and UMR106, and important features, such as tyrosine phosphorylation and activation of signalling mechanisms, have been characterized (Barrio and Etcheverry 2006; Cortizo and Etcheverry 1995; Salice et al. 1999). Although repetitively studied in mammals, vanadate effects have not been investigated in marine vertebrates (e.g. fish and amphibians), despite being recognized as the second most abundant transition metal in seawater (Rehder 2003).

In this paper, we describe the effects of two different vanadate solutions on the growth and mineralization performances of a gilthead seabream bone-derived cell line (VSa13) recently developed in our laboratory (Pombinho et al. 2004) and capable of mineralizing its extracellular matrix.

Materials and methods

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), antimycotic (fungizone), trypsin-EDTA solution and L-glutamine were purchased from Invitrogen. Tissue culture dishes were purchased from

Sarstedt or Nunc. CellTiter 96 non-radioactive proliferation assay kit was purchased from Promega. All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

Cell culture maintenance

Cultured cells were maintained in DMEM supplemented with 1% penicillin-streptomycin, 1% fungizone, 2 mM L-glutamine and 10% FBS, and incubated at 33°C in a 10% CO_2 humidified atmosphere. Confluent cell cultures were divided (1:2) every 3–4 days using trypsin–EDTA solution.

Vanadate solution preparation

Metavanadate stock solution (50 mM, pH 6.7) was prepared from ammonium metavanadate (NH_4VO_3). Decavanadate stock solution was obtained by adjusting the pH of the former solution to 4.0, as described elsewhere (Aureliano and Madeira 1994). The acquired characteristic orange color in decavanadate solution upon acidification reveals the presence of decameric vanadate species (V_{10}). Decavanadate stock solution was always adjusted to pH 7.0 immediately before using it. Both vanadate solutions were stored at 4°C. Although total vanadate concentration does not change, note that in case of decavanadate solutions the concentration in decameric vanadate species was effectively reduced 10-fold, meaning that stock decavanadate solutions contain 5 mM decameric vanadate species. However, vanadate concentrations are always given for monomeric vanadate, i.e. total vanadate.

Characterization of vanadate solutions

The composition of both vanadate solutions was analysed by ^{51}V Nuclear Magnetic Resonance (NMR) spectroscopy, as described elsewhere (Aureliano and Madeira 1994). Briefly, the composition of vanadate solutions upon dilution into DMEM was analysed in a Bruker AM-400 spectrometer at 105.2 MHz equipped with a 5-mm multinuclear inverse probe, using a 90° pulse Fourier transform technique. Spectra were acquired at room temperature using 0.5 ml of vanadate samples in the medium, containing at least 10% D_2O, under the following conditions: spectral width 45,455 Hz, accumulation time 0.05 s and relaxation delay 0.01 s. ^{51}V NMR chemical shifts are reported relative to an external reference of $VOCl_3$ (0 ppm). The relative areas of the several free or/and bound vanadate resonances were integrated and the line widths were obtained after subtracting the value (20 Hz) used in line broadening. The concentration of each vanadate oligomer V_x was calculated from the fractions of the total integrated areas observed in the recorded spectra as described (Equation 1). The symbol A corresponds to the area measured for the x vanadate species with the n aggregation number (number of vanadium atoms), A_t, the sum of measured areas and $[V_t]$ corresponds to total vanadate concentration.

$$[V_x] = \frac{A_x}{A_t} \times \frac{[V_t]}{n} \quad (1)$$

The calculated concentrations of vanadate oligomers were reproducible within 2–4%. For quantitative measurements, all spectra parameters were kept constant. Vanadate concentrations are always given for monomeric vanadate, i.e. total vanadium.

Unlike metavanadate, decavanadate solutions are unstable. The partial deoligomerization of decameric vanadate species present at the decavanadate solutions upon dilution in DMEM medium was analysed by ultraviolet/visible spectroscopy at 400 nm, as described elsewhere (Soares et al. 2006).

Cell viability measurement

Cytotoxic and proliferative effects of vanadate oligomers on VSa13 cells were assessed through cell viability analysis using the CellTiter 96 non-radioactive proliferation assay kit according to the manufacturer's instructions. Tetrazolium reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; inner salt, MTS) and the electron-coupling reagent, phenazine methosulfate, were mixed in solution in a 20:1 proportion. Once added to cell culture, MTS is reduced into formazan by the dehydrogenase enzyme found in metabolically active cells. Formazan formation, which is proportional to the number of living cells (Malich et al. 1997), was followed at 490 nm in 96-well tissue culture dishes. Incubation with 20 µl of reagent mixture during 1 h was determined to be the optimal conditions for VSa13 cell viability measurement. Vanadate cytotoxic effects were studied in confluent cell cultures (approximately 1.7×10^4 cells/well) while effects on cell proliferation were investi-

gated in low-density cell cultures (starting from 1.5×10^3 cells/well).

Vanadium accumulation measurement

Cells were cultured for 0, 1, 2, 4, 8 and 24 h in the presence of 10 μM metavanadate or decavanadate. At appropriate times, cell cultures were washed three times with phosphate-buffered saline (PBS), supplemented with 1 ml of MilliQ water (Millipore) and placed at −80°C for 10 h. Frozen cells were thawed at room temperature for 15 min, collected using a cell scraper and transferred into a 2-ml microcentrifuge tube. Cell culture dishes were washed once with 1 ml of MilliQ water and washing solution was transferred into the microcentrifuge tube containing the initial cell extract. Total protein content was quantified from cell extracts using Bradford reagent. Samples were then digested at 100°C with 30 μl of 65% HNO_3 and final solid residues dissolved in 5% HCl solution. Vanadium concentration was determined by atomic absorption spectrometry analysis using a GBC Avanta atomic absorption spectrometer equipped with a GBC GF 3000 graphite furnace system and a GBC PAL 3000 auto sampler working at a furnace program of 63 s with an argon gas flow of 3.0 L/min. The vanadium lamp was operated at 318.2 nm, with slit width of 0.2 nm, and the instrument was calibrated against a series of solutions containing 12.5, 25, 37.5 and 50 ppb of vanadium. Calibrating standards were obtained by successive dilutions of a standard solution of vanadium 1002±2 mg/L (Merck). The detection and quantification limits of the instrument for these analysis conditions, determined according to ISO 8466-1, were 5 and 11 ppb, respectively.

Extracellular matrix mineralization and nodule detection

Cells were seeded in 24-well plates at 2×10^4 cells/well and cultured in DMEM supplemented with 10% FBS. To induce ECM mineralization, confluent cultures received medium supplemented with 50 μg/mL of L-ascorbic acid (vitamin C), 10 mM β -glycerophosphate and 4 mM $CaCl_2$ up to 4 weeks. Wherever appropriate, cells were washed three times with PBS at 4°C, fixed with 10% formaldehyde (in PBS) for 1 h at 4°C, washed three times with distilled water, then incubated with 5% silver nitrate for 30 min under ultraviolet light. Relative levels of ECM mineralization were determined by densitometric methods using Quantity 1 software (Bio-Rad).

Statistical analysis

The data were presented as average and standard deviation of measurements taken at least in three separate experiments. Statistical significance of data was analysed wherever indicated by Student *t* test or analysis of variance (ANOVA) analysis. Differences were considered to be significant at $P<0.05$.

Results

Vanadate solutions characterization in DMEM

^{51}V-NMR spectroscopy of 1 and 5 mM decavanadate solutions in DMEM, at pH 7.0, revealed three signals attributed to three specific vanadium atoms in V10 structure: V10A at −515 ppm, V10B at −498 ppm and V10C at −425 ppm (Fig. 1a). Conversely, in 1 and 5 mM metavanadate solutions (Fig. 1a), detected signals ascribed to mono- (V1), di- (V2), tetra- (V4) and pentameric (V5) vanadate species, respectively, at −556 ppm, −571 ppm, −579 ppm and at 587 ppm (Fig. 1a). Monomeric NMR signal is clearly broadened, with a half line-width value (270 Hz) approx. 3.4-fold of normal value (80 Hz), probably caused by vanadate interactions with compounds present in DMEM such as glucose (25 mM) and piruvate (1 mM), or even interactions with several proteins also present in the medium. For each metavanadate concentration in DMEM (1 and 5 mM), different amounts of mono (V1), di (V2), tetra (V4) and pentameric (V5) species have been observed, that corresponded to different species profiles (Fig. 1b). While tetrameric species were predominant at 5 mM metavanadate, monomeric vanadate was the major specie in the lower concentration (Fig. 1b). Conversely, decameric vanadate species present in the decavanadate solutions increased linearly with total vanadate concentration (Fig. 1c).

Although ^{51}V-NMR spectroscopy is an essential technique for the analysis of vanadate oligomers composition and interactions, approximately 1 h acquisition time was necessary to obtain a clear spectrum of 1 mM decavanadate solution (Fig. 1a).

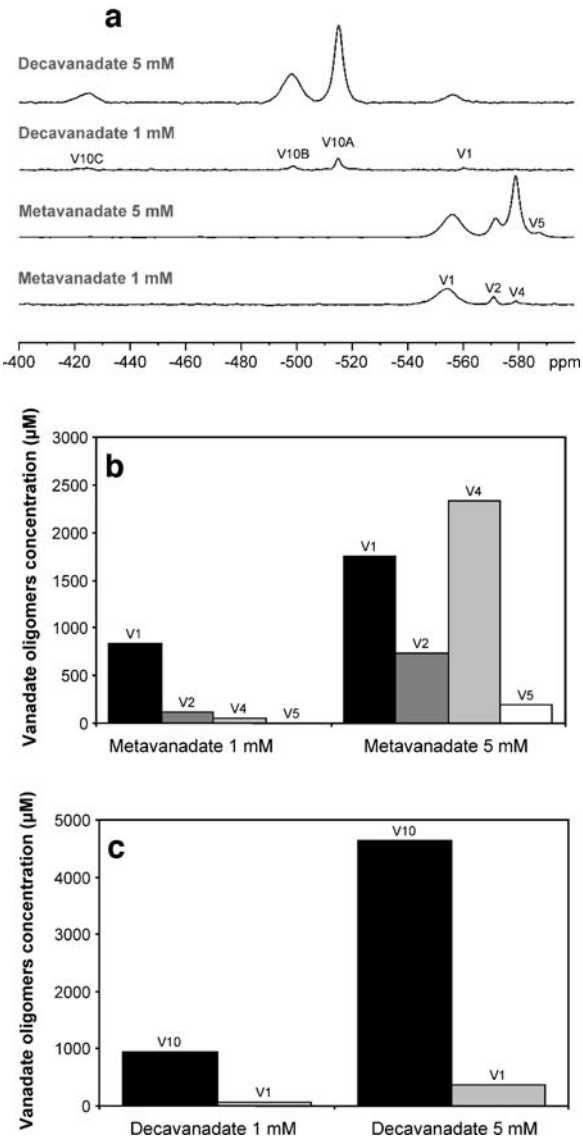

Fig. 1 105.2 MHz ^{51}V NMR spectra, at 22°C, of two vanadate concentrations (total vanadate), namely, 1 and 5 mM of decavanadate and metavanadate in DMEM, pH 7.1. (**a**) V1 and V2 signals correspond to monomeric ($H_2VO_4^-$) and dimeric ($HV_2O_7^{3-}$ and $H_2V_2O_7^{2-}$) vanadate species, respectively, irrespective of the protonation state, whereas V4 and V5 correspond to cyclic tetrameric ($V_4O_{12}^{4-}$) and pentameric ($V_5O_{15}^{5-}$) vanadate species. V10A, V10B and V10C signals correspond, respectively, to the V(2), V(1) and V(3) vanadium atoms in the decameric vanadate specie ($V_{10}O_{28}^{6-}$). The concentrations of each vanadium (V) species for metavanadate (**b**) and decavanadate (**c**) solutions were calculated from the fractions of the total integrated areas observed in the spectra. For quantitative measurements, spectral parameters were kept constant and the spectra were normalized regarding 5 mM decavanadate spectra

In this sense, ^{51}V-NMR spectroscopy is not adequate to follow the decomposition kinetics of decavanadate, in particular for vanadate concentrations lower than 1 mM. However, the disappearance of decameric vanadate species can be easily followed by ultraviolet/visible (UV/Vis) spectroscopy. Therefore, the stability of decameric species in DMEM was analysed by UV/Vis, at 33°C, using a 250 μM decavanadate solution (total vanadium concentration, meaning 25 μM decameric vanadate species). It was observed that in DMEM, decameric species decompose through a first-order kinetic, with a half-life time of 150 min (not shown).

Long- and short-term exposure to meta- and decavanadate

Cytotoxicity of vanadate oligomers on VSa13 cells was tested in confluent cultures for either 15 days using concentrations ranging from 0 to 250 μM (chronic toxicity) or 4 h using a concentration of 1 mM (acute toxicity). Prolonged exposure to meta- or decavanadate similarly affected VSa13 cell viability (Fig. 2): treatments with concentrations up to 7.5 μM did not decrease cell viability (a slight increase was even observed after 15 days), while treatments with concentrations from 10 to 250 μM seriously affected cell viability, killing most of the cells at highest concentrations. On the contrary, short-term exposure to meta- and decavanadate differentially affected VSa13 cell viability (Fig. 3): While 1 mM decavanadate significantly reduced cell viability after 2 hours, 1 mM metavanadate showed no effects. After 4 hours, both solutions significantly (according to ANOVA analysis) reduced the cell viability to levels approximate to 50%. This observation suggests that decameric vanadate species, still present in culture medium after 2 h, are likely to induce more toxicity effects than monomeric vanadate species, certainly the most abundant vanadate form found in culture medium after 4 h (half-life time of decavanadate in DMEM was estimated at 2.5 h) upon decameric vanadate disintegration.

Stimulation of cell proliferation by vanadate

Vanadate effect on VSa13 cell proliferation rate was also investigated in dividing cultures (Fig. 4), and both metavanadate and decavanadate exhibited similar effects (thus, only metavanadate results are shown), *i.e.* a significant stimulation of proliferation rate was observed for 7.5 μM concentration (accord-

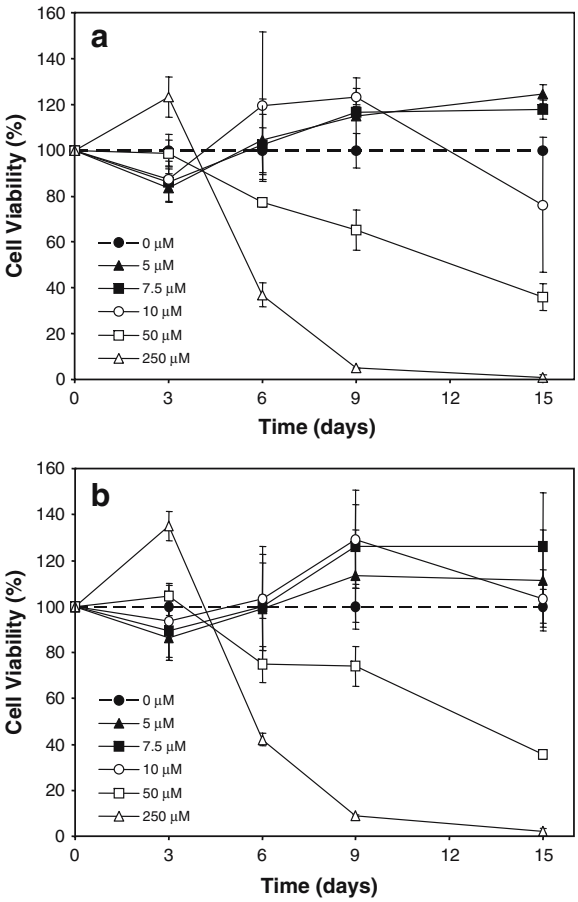

Fig. 2 Long-term effect of metavanadate and decavanadate on VSa13 cell viability. VSa13 cells were seeded in 96-well plates, grown in DMEM until confluence then treated with metavanadate (**a**) and decavanadate (**b**) using concentrations ranging from 5 to 250 μM. Cell viability was evaluated at appropriate times using MTS assay and is presented as the percentage of control value (set to 100%). Values are the mean of at least three independent experiments

ing to ANOVA analysis). On the contrary, 10 nM insulin (concentration used in mammalian bone-derived cell line, Quarto et al. 1992), had no effect on cell proliferation (Fig. 4) suggesting that vanadate, despite proven insulin-mimetic properties, stimulates cell proliferation. Nevertheless, no effects were observed in cell proliferation using insulin concentrations up to 100 nM (not shown).

Metavanadate and decavanadate exhibit different rates of accumulation in cells

The amount of vanadium accumulated within VSa13 cells was determined in confluent cultures treated with 10 μM meta- or decavanadate for 0, 1, 2, 4, 8 and 24 h after subtraction of vanadium content measured in non-treated cells (2.54±0.43 μg V/g of protein) (Fig. 5). After 1 h of treatment, vanadium accumulation was higher in cells treated with metavanadate (4.61±0.29 μg V/g of protein) than in cells treated with decavanadate (3.24±0.24 μg V/g of protein), being the effect statistically significant (according to Student's *t* test). A similar, but more pronounced, difference between meta- and decavanadate-related vanadium accumulations was observed after 2 h of treatment (6.85±0.4 μg and 3.95±0.1 μg V/g of protein, respectively). Longer incubation times resulted in a higher accumulation of vanadium (approximately 18-fold increase above control value after 24 h), but no significant differences were observed between metavanadate and decavanadate treated cells, as expected. These results suggest that monomeric species enter VSa13 cells and accumulate more easily than decameric species and that accumulation rates become similar only when most of decameric molecules are decomposed (half-life time of decavanadate in DMEM was estimated at 2.5 h).

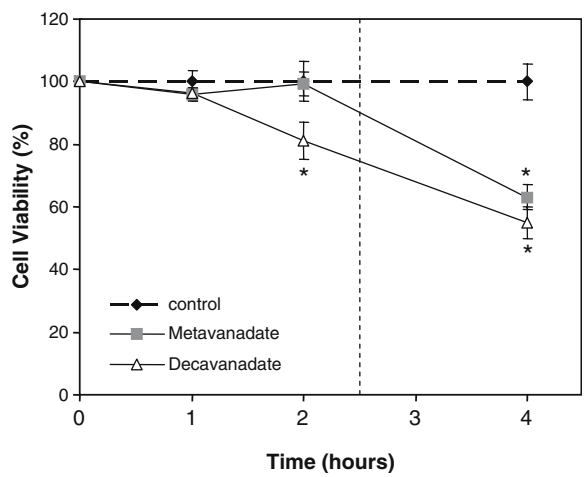

Fig. 3 Short-term effect of metavanadate and decavanadate on VSa13 cell viability. VSa13 cells were seeded in 96-microwell plates, grown in DMEM until confluence then treated with 1 mM metavanadate and decavanadate. Cell viability was evaluated at appropriate times using MTS assay and is presented as the percentage of control value (set to 100%). Values are the mean of at least three independent experiments. *Asterisk* indicates that values are statistically different in comparison to the respective control ($P<0.05$ one-way ANOVA). *Dashed line* indicates the half-life of decavanadate in DMEM estimated at 2.5 h

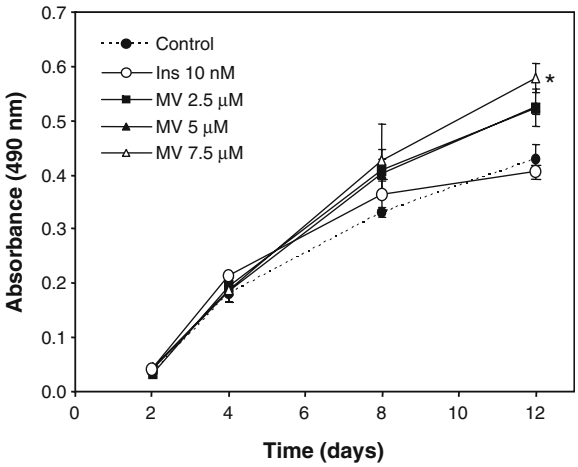

Fig. 4 Effect of metavanadate and insulin on VSa13 cell proliferation. VSa13 cells were seeded in 96-well plates at 1.5×10^3 cell/well then treated with metavanadate using concentrations ranging from 2.5 to 7.5 μM or 10 nM insulin. Cell proliferation was evaluated at appropriate times using MTS assay. Values are the mean of at least three independent experiments. *Asterisk* indicates that values are statistically different in comparison to the respective control ($P<0.05$ one-way ANOVA)

Vanadate and insulin decrease ECM mineralization

Vsa13 cells were cultured under mineralizing conditions for 4 weeks. Then, extracellular matrix mineralization was evaluated by densitometric analysis of von Kossa staining. The effects of vanadate (5 μM) and insulin (10 nM) on ECM mineralization were tested separately or in combination (Fig. 6). Vanadate 7.5 μM concentration was excluded from this experiment because of cytotoxic effects observed after 4 weeks of treatment (data not shown). Insulin, metavanadate and decavanadate significantly decreased ECM mineralization (according to ANOVA analysis), although to different extents: 31%, 74% and 78% decrease for insulin, meta- and decavanadate, respectively. The combination of insulin with any of the vanadate oligomers resulted in a more pronounced decrease (88% and 86%, respectively), although not significant (according to ANOVA analysis), suggesting an additive effect. A lower concentration of vanadate (2.5 μM) was also tested and shown to exhibit a similar, but less pronounced, decrease (data not shown) indicating a dose-dependent inhibition of ECM mineralization by vanadate oligomers.

Discussion

The effects of vanadium on bone biology have been extensively studied in several mammalian *in vivo* and *in vitro* systems (Etcheverry and Cortizo 1998; Shechter 1990), but, to our knowledge, no studies have been performed using bone-derived systems of aquatic vertebrate origin. In this study, we analyse, for the first time, the effects different vanadate oligomers have on bone-derived cell line mineralization. However, the composition of vanadate solutions is very sensitive to vanadate concentration, pH, buffer and other compounds used in biochemical studies. Therefore, it is of primary importance to precisely characterise the species that can interact with a system before attempting to understand the effects promoted by vanadate solutions. Unlike metavanadate containing several oxovanadates, decavanadate solutions contain decameric vanadate species (V10). However, V10 is unstable and decomposes into monomeric vanadate with a half-life time of above 2 h in DMEM. In this regard, effects of decavanadate were always compared with those of metavanadate.

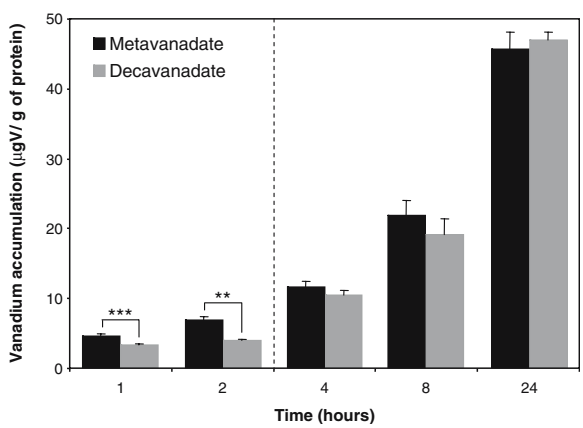

Fig. 5 Vanadium accumulation in VSa13 cells upon vanadate solutions exposure. VSa13 cells were seeded in 100-mm plates, grown in DMEM until confluence then treated with 10 μM metavanadate or decavanadate. At appropriate times, vanadium content was determined by atomic absorption from cellular extract and normalized with protein content. Vanadium content measured in non-treated samples (2.54±0.43 μg V/g of protein) was subtracted from each value. *Asterisks* indicate statistically significant differences between metavanadate and decavanadate according to Student's test (**$p<0.005$ and ***$p<0.001$). Values are the mean of at least three independent experiments. *Dashed line* indicates the half-life of decavanadate in DMEM estimated at 2.5 h

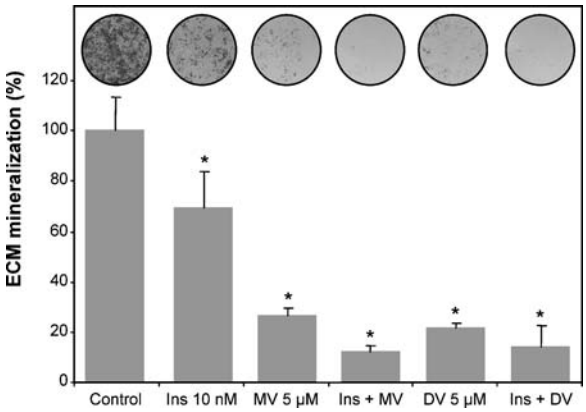

Fig. 6 Effect of metavanadate, decavanadate and insulin on extracellular matrix mineralization. VSa13 cells were seeded in 24-well plates, grown in DMEM until confluence then treated for mineralization. Mineralizing cultures were subsequently treated with 10 nM insulin (Ins), 5 μM metavanadate (MV), 5 μM decavanadate (DV), metavanadate and insulin (Ins+MV), decavanadate and insulin (Ins+DV) or left untreated. Mineral deposition was revealed by von Kossa staining and evaluated by densitometric analysis. Pictures of von Kossa-stained VSa13 cells are presented above each treatment. Values are the mean of at least three independent experiments. *Asterisk* indicates that values are statistically different in comparison to the respective control ($P<0.05$ one-way ANOVA)

Monomeric and decameric vanadate species affect VSa13 cells differently

Prolonged exposure to low levels of meta- and decavanadate similarly affected VSa13 cell viability and proliferation, whereas short-term exposure to high levels of decavanadate was more toxic than that of metavanadate. In DMEM, decavanadate decomposes into monomeric species with a half-life time of ~150 min, suggesting that its long-term effects are most probably caused by newly formed monomeric species (as for metavanadate), whereas short-term effects are likely caused by decameric vanadate species. Considering that decavanadate solution contains 10 times less molecules (particularly in shorter exposure times, in which decameric species are predominant) than metavanadate (in which monomeric species are predominant), short-term toxicity of decavanadate is even more remarkable. This result is consistent with previous observations that decavanadate and metavanadate produce different oxidative stress levels in cardiac muscle in toadfish and gilthead seabream (Aureliano et al. 2002; Soares et al. 2007) and interact differently *in vitro* with specific proteins such as myosin and sarcoplasmic reticulum Ca^{2+}-ATPase (Aureliano and Madeira 1994; Tiago et al. 2004; Aureliano and Gândara 2005). Prolonged exposure to low levels of vanadate resulted in an apparent increase in cell viability/number, an effect that was previously observed in mammalian bone-derived cell cultures (MC3T3E1 and UMR106) (Barrio et al. 1997; Cortizo et al. 2000; Etcheverry et al. 1997; Salice et al. 1999) and attributed to its ability to increase phosphotyrosine protein levels and to inhibit phosphotyrosine phosphatases, thus regulating growth signalling pathways.

Although cellular accumulation of metavanadate has been previously demonstrated in mammalian systems, *e.g.* bovine kidney and Caco-2 cells (Bracken et al. 1985; Yang et al. 2004), this process has never been analysed in a comparative study with decavanadate. We present evidences that vanadium differently accumulates in VSa13 cells treated with metavanadate or decavanadate solutions (decavanadate treatments resulted in significantly lower vanadium accumulation at 1 and 2 h; Fig. 5), which was probably caused by an inexistent or a less efficient uptake mechanism for decameric species.

Vanadate stimulates VSa13 cell proliferation

Both vanadate oligomers exhibited a similar effect on VSa13 growth rate, *i.e.* a stimulation of cell proliferation. A similar effect has been reported in mammalian bone-derived cell lines and associated with the activation of the insulin receptor. Insulin was shown not to affect VSa13 cell proliferation, indicating that either insulin receptor/signalling pathway is absent in VSa13 cells (this hypothesis was later ruled out after insulin was shown to affect ECM mineralization) or vanadate stimulating effect is not mediated through insulin signalling pathway. Several studies have already proposed cytosolic tyrosine kinase receptor as a mediator of vanadate proliferative effect (Shisheva and Shechter 1992, 1993), and we propose that a similar mechanism could occur in VSa13 cells.

Inhibition of ECM mineralization by vanadate

Mineralization of VSa13 extracellular matrix was mildly reduced by insulin, strongly inhibited by vanadate, regardless of its oligomerization (monomeric or decameric), and even more strongly inhibited

when both insulin and vanadate were present. Two important regulatory effects have been associated with vanadium osteogenic action in mammalian bone-derived cells: the inhibition of alkaline phosphatase activity and the stimulation of type I collagen synthesis (Barrio and Etcheverry 2006; Canalis 1985b; Lau et al. 1988). Both processes are essential to the correct mineralization of the extracellular matrix of bone cells (Murshed et al. 2005), and it is likely that vanadium effect on vertebrate bone depends on the equilibrium between its stimulatory and inhibitory properties. Vanadium derivatives were previously shown to specifically activate insulin-associated pathways (i.e. MAPK and PI-3K pathways) through phosphorylation of common intermediates such as extracellular signal-regulated kinase (ERK) (Barrio and Etcheverry 2006). Moreover, vanadium proliferative effect was reverted by specific inhibitors of insulin-associated pathways. In one of those studies, vanadyl (IV)-ascorbate, a vanadium complex, significantly increased the rate and extent of mineral deposition within the ECM of mouse MC3T3-E1 bone-derived cells and stimulated type I collagen synthesis (Cortizo et al. 2006). These effects were apparently related to both MAPK and PI-3K pathways activation and ERK increased phosphorylation. In another study, it was described that the alkaline phosphatase activity is inhibited in MC3T3-E1 cells by a trehalose vanadyl complex independently of regular insulin-associated pathways. Based on published results, we suggest that vanadate inhibitory effect on VSa13 ECM mineralization could be associated with either direct interference of vanadium into key processes such as alkaline phosphatase activity, or indirect interference of vanadium into insulin-associated pathways. Recently, it was shown that the ECM mineralization of MC3T3-E1 cells is inhibited by platelet-derived growth factor through ERK activation (Kono et al. 2007). This result is, on one hand, contradictory to a previous observation where ECM mineralization of MC3T3-E1 cells was stimulated by vanadium through ERK activation (Cortizo et al. 2006), but, on the other hand, in total agreement with the inhibition of VSa13 ECM mineralization by vanadate, possibly through ERK activation. The characterisation of the mechanisms involved in vanadate inhibitory effect in VSa13 cell line will need to be investigated in future studies.

Conclusion

Vanadate was shown here to accumulate within VSa13 bone-derived cells, stimulating growth performance through insulin-independent mechanisms and preventing ECM mineralization probably through multiple processes involving regulation that may or may not depend on the activation of insulin-stimulated pathways. The presence of decameric vanadate species was also shown to produce differences (accumulation and cytotoxicity) between meta- and decavanadate in short-term exposure while acting similarly in prolonged exposure caused eventually by the decomposition of decavanadate into other oligomers. VSa13 cells were shown to be a suitable model to study vanadium mechanisms of action in vertebrate bone development and to be a good alternative to mammalian systems.

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