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Vanadate proliferative and anti-mineralogenic effects are mediated by MAPK and PI-3K/Ras/Erk pathways in a fish chondrocyte cell line

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Abstract **We recently reported proliferative and anti-mineralogenic effects of vanadate on fish chondrocytes and here we investigate the signalling pathways associated with these effects. Our data show that vanadate stimulates chondrocyte proliferation through the MAPK pathway, using signalling mechanisms similar to those used by IGF-1, while it inhibits chondrocyte differentiation/mineralization through a putative PI-3K/Ras/Erk signalling, a pathway shared with insulin. Our data also suggest that vanadate impairs ECM mineralization not only by interfering with regulatory pathways but also by inhibiting enzymatic activity of ALP. Finally, this work provides additional evidence for the conservation, throughout evolution, of mechanisms regulating chondrocyte proliferation and differentiation.**

Keywords: Vanadium; Insulin-like activity; Intracellular signalling; In vitro mineralization; Bony fish; Bone-derived cell line

1. Introduction

Vanadium, mostly known for enhancing mechanical properties of alloy steels in the metal industry, has also been recognized as an essential trace element for biological activity in most living animals [1]. In mammals, vanadium has been associated to a variety of insulin-like effects [2] and its action associated to the specific regulation of protein tyrosine phosphatases and consequent activation of tyrosine kinase receptors, including the insulin receptor [2,3]. A role for vanadium in bone formation has been demonstrated [4,5] but mechanisms of action have remained largely unexplored. In vitro studies, using calvaria primary cultures [6,7] and bone-derived cell lines [5,8,9], have evidenced the regulation of bone-related enzymes activity (e.g. alkaline phosphatase (ALP) and protein tyrosine phosphatases), the stimulation of bone-related protein synthesis (e.g. type I collagen), the alteration of bone-related cell proliferation and extracellular matrix (ECM) mineralization, and the activation of insulin and insulin-like growth factor 1 (IGF-1) signalling mechanisms [5,9] by vanadium compounds. However, there is still much to unveil in order to understand vanadium mechanisms of action in bone.

In this study, wortmannin, a specific inhibitor of phosphatidyl inositol-3 kinase (PI-3K) pathway, and PD98059, a specific inhibitor of mitogen-activated protein kinase (MAPK) have been tested for their effects on vanadium-associated alteration of growth performances, ECM mineralization, ALP activity, collagen synthesis in VSa13 cells.

2. Materials and methods

2.1. Cell culture and ECM mineralization

VSa13 cells were cultured as described previously [10]. ECM mineralization was induced in confluent cultures by supplementing medium with 50 μg/ml of L-ascorbic acid, 10 mM β-glycerophosphate and 4 mM $CaCl_2$. At appropriate times, mineral deposition was revealed using the von Kossa staining method and quantified by densitometric analysis [10]. Culture medium was renewed every 2 days in proliferation experiments and every 3.5 days in mineralization experiments. Insulin (1–100 nM), IGF-1 (1–100 nM) and vanadate (2.5–7.5 μM) treatments were applied at the time of medium renewal.

2.2. Preparation of vanadate, PD98059, wortmannin, insulin and IGF-1 stock solutions

Vanadate solution (50 mM, pH 6–7) was prepared as described previously [8]. PD98059 and wortmannin were solubilised in DMSO at 6 and 10 mg/ml, respectively. Bovine insulin (Sigma–Aldrich) and *Pagrus auratus* IGF-1 (Novozymes GroPep) were solubilised in pH 2 water at 10 μM and in pH 6 water at 0.1 mg/ml, respectively. Milli-Q water (Millipore) was used to prepare vanadate, insulin and IGF-1 solutions.

2.3. Measurement of cell proliferation

VSa13 cell proliferation was determined from cultures seeded in 96-well plates at 1.5×10^3 cells/well using the CellTiter 96 non-radioactive proliferation assay kit (Promega). At appropriate times, cells were incubated for 1 h with 20 μl of reagent mixture then cell proliferation was determined from absorbance at 490 nm.

2.4. Measurement of ALP activity

VSa13 cells grown for 4 weeks under mineralizing conditions were washed three times with phosphate-buffered saline (PBS), scrapped off the plate into 500 μl of 0.1% Triton X-100 then centrifuged for 1 min at 16000 × *g*. ALP activity was determined at 37 °C from initial rates of *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis into *p*-nitrophenol (*p*-NP). Formation of *p*-NP was monitored at 405 nm from the following reaction mixture: 800 μl of reaction buffer (55 mM glycine and 0.55 mM $MgCl_2$ at pH 10.5), 100 μl of 5 mM *p*-NPP and 100 μl of cell extract supernatant. ALP activity was normalized with protein content determined using Bradford reagent (Bio-Rad).

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Abbreviations: ALP, alkaline phosphatase; ECM, extracellular matrix; IGF-1, insulin-like growth factor 1; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidyl inositol-3 kinase

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2.5. Measurement of collagen content

VSa13 cells grown for 4 weeks under mineralizing conditions were washed three times with PBS then stained with Sirius red dye (F3BA; Chroma) as described previously [11]. Bound dye was solubilised into 200 μl of 0.1 N NaOH and collagen content was determined from absorbance at 550 nm.

2.6. Statistical analysis

Values are the mean of at least three separate experiments and are presented with their standard deviation. Differences upon treatment were analyzed by one-way ANOVA and considered significant at $P < 0.05$.

3. Results and discussion

In vitro studies investigating vanadium insulin-like effects and specific targets in bone have been largely restricted to a particular taxonomic group (i.e. mammals) and to particular cell types (i.e. osteoblast-like and osteosarcoma cells). This work evidences, for the first time in a non-mammalian in vitro system and using a chondrocyte-like cell line, the role of MAPK and PI-3K pathways in the mediation of vanadate effects on proliferation and mineralization.

3.1. MAPK pathway mediates vanadate stimulation of VSa13 cell proliferation

Vanadate (2.5–7.5 μM), insulin (1–100 nM) and IGF-1 (1–100 nM) were tested for their effect on VSa13 cell growth. Both vanadate and IGF-1, but not insulin, stimulated cell proliferation to a similar extent (strongest stimulation at 7.5 μM and 10 nM, respectively; Supplementary Figures 1 and 2 and Fig. 1). Wortmannin and PD98059, known inhibitors of PI-3K and MAPK pathways, respectively, were then tested for their effect on VSa13 cell proliferation and for their capacity of reverting IGF-1 and vanadate stimulatory effects. Dividing cultures of VSa13 cells were treated for 8 days with 0.1 μM wortmannin, 100 μM PD98059 (non-toxic concentrations, data not shown) in the presence or absence of insulin, vanadate or IGF-1 (Fig. 1). PD98059, but not wortmannin, totally abolished vanadate and IGF-1 stimulation of cell proliferation (inhibitors alone had no effect) suggesting that proliferation of VSa13 cells is stimulated by vanadate through the activation of the MAPK pathway. A similar contribution of the MAPK pathway in the transduction of vanadate proliferative effect has been reported in mammalian osteoblast-like cells [12,13], suggesting a conservation of mechanisms of action throughout evolution and across bone cell types. The inability of insulin to enhance VSa13 cell proliferation suggests that the activation of MAPK pathway by vanadate would occur through an insulin receptor-independent manner. Similarly, the stimulation of VSa13 cell proliferation by IGF-1 through MAPK pathway and to a similar extent, suggest that the activation of MAPK pathway by vanadate could occur through IGF-1 receptor and/or signalling pathway, although we cannot exclude that other tyrosine kinase receptors (e.g. platelet-derived growth factor (PDGF) receptor) may be involved [5]. Similar mechanisms (i.e. those related to the proliferative effect of IGF-1) have been recently reported in the mouse chondrocyte ATDC5 cell line [14] and thus, based on those results as well as our data reported in this study, we propose the existence of a mechanism involving MAPK pathway that would regulate chondrocyte proliferation and transduce vanadate effect (Fig. 5A).

3.2. Putative PI-3K/Ras/Erk pathway mediates vanadate impairment of VSa13 ECM mineralization

Wortmannin and PD98059 were then tested for their effect on VSa13 ECM mineralization and for their capacity of reverting vanadate and insulin inhibitory effects previously demonstrated [8] and further confirmed here. Mineralizing VSa13

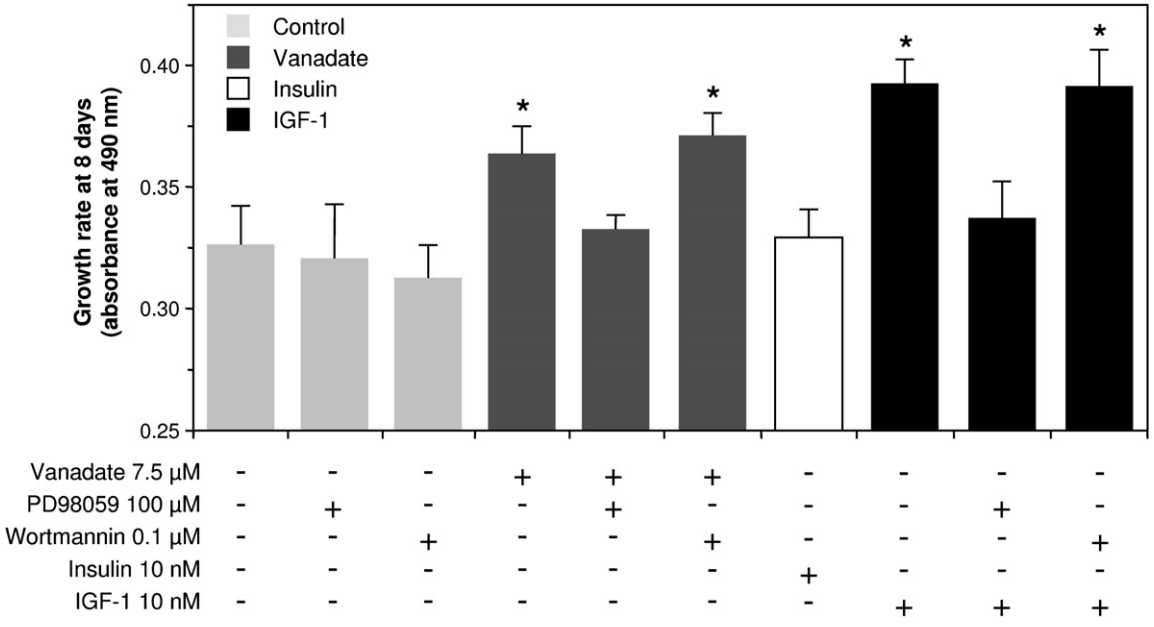

Fig. 1. Effect of vanadate, insulin, IGF-1, wortmannin and PD98059 on VSa13 cell proliferation. VSa13 cells were seeded in 96-well plates at 1.5×10^3 cells/well then either left untreated or treated with PD98059, wortmannin, vanadate, insulin or IGF-1, alone or in combinations. Cell proliferation was evaluated after 8 days using MTS assay. Asterisk indicates values statistically different from corresponding controls ($n \geqslant 3$; $P < 0.05$; one-way ANOVA).

cultures were treated for 4 weeks with 0.1 μM wortmannin, 100 μM PD98059, 10 nM IGF-1, 10 nM insulin, and/or 5 μM vanadate, then evaluated for mineralization. While unaltered by IGF-1, mineral deposition was increasingly impaired by insulin (1.4-fold), vanadate (8-folds) and almost abolished by a combination of both agents (Fig. 2). Apparent synergy between vanadate and insulin could suggest that different signalling pathways are involved in both mechanisms of action, or that vanadate potentiates insulin action during mineralization, e.g. by inhibiting PTPases, therefore increasing sensitivity of insulin pathway as previously suggested [15]. Interestingly, ECM of PD98059-treated cells, but not that of wortmannin-treated cells, exhibited a significant increase in mineral deposition (3.2-folds), suggesting a down-regulation of ECM mineralization by MAPK pathway. We propose, in agreement with recent results obtained in mouse ATDC5 chondrocyte cells [14,16], that MAPK pathway may affect ECM mineralization of VSa13 cells by inhibiting cell differentiation. The role of MAPK pathway in regulating VSa13 ECM mineralization was further confirmed by the reversion (100% and 72.5%, respectively) of insulin and vanadate anti-mineralogenic effects with PD98059. Surprisingly, wortmannin also reverted (100% and 57.5%, respectively) insulin and vanadate anti-mineralogenic effects, suggesting a role for PI-3K pathway in regulating VSa13 ECM mineralization. As expected, impairment of mineralization by the combined effects of insulin and vanadate was reverted to an extent similar to that observed for vanadate alone. Altogether, these results suggest that both MAPK and PI-3K pathways could mediate vanadate and insulin anti-mineralogenic effects. We propose that regulation of VSa13 cell differentiation/mineralization is exerted through the PI-3K/Ras/Erk pathway (a combination of MAPK and PI-3K pathways), a finding that is in agreement with recent results obtained for mouse MC3T3-E1 cells [17]. We further propose, based on overall available data as well as results reported in this study, that a similar mechanism is responsible for transducing vanadate effects (Fig. 5B). However, incomplete reversion of vanadate anti-mineralogenic effect by both PD98059 and wortmannin (83.2% reversion, result not shown) suggests that other differentiation/mineralization-related mechanisms are affected by vanadate. ALP, by cleaving pyrophosphate, an inhibitor of ECM mineralization [18], and collagen, an essential structural component of ECM [19], were considered as putative targets for vanadate action and further investigated.

3.3. ALP activity is inhibited while collagen content is increased by micromolar concentrations of vanadate

Enzymatic activity of ALP was first determined in mineralized cell cultures treated with 5 μM vanadate and/or 10 nM insulin. Interestingly, a 40% increase in ALP activity was observed following ECM mineralization, while activity remained similar to non-mineralizing control cells upon vanadate treatment (Fig. 3). In similar experiments, collagen content was shown to increase by 57% in mineralized cultures and by an additional 17% when those cultures were also treated with vanadate (Fig. 4). The stimulation of both ALP activity and collagen production in mineralized cultures is consistent with their role in mineralization mechanisms. The inability of insulin to affect ALP activity and collagen production suggests that vanadate action occurs through an insulin receptor-independent manner. ALP activity was then measured in cellular extracts of mineralized cultures (4 weeks) in the presence of increasing concentrations of vanadate: activity was stimulated at low concentration (5 μM) while strongly inhibited at higher concentrations (from 25 to 500 μM) of vanadate. We propose, in

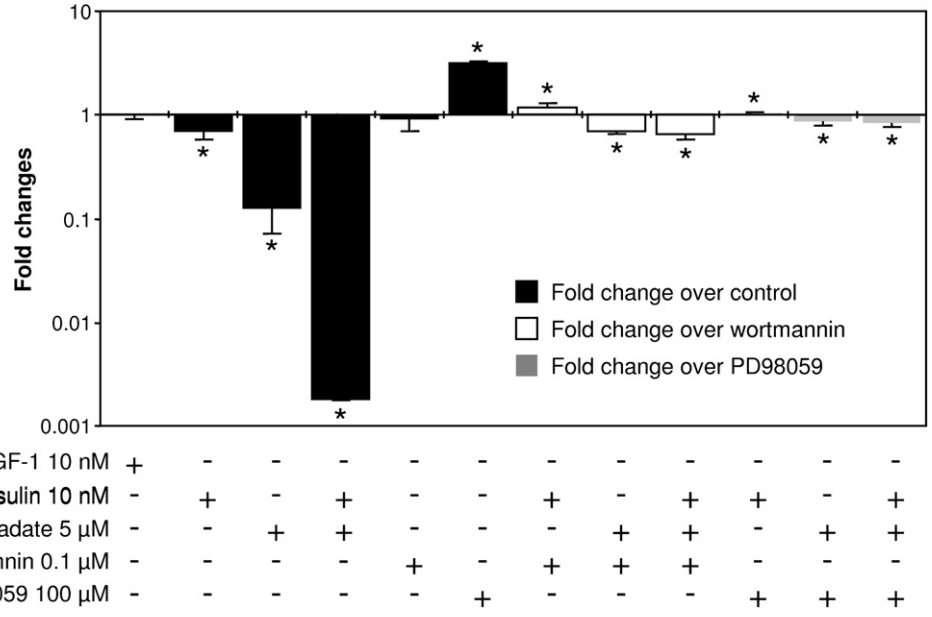

Fig. 2. Effect of IGF-1, vanadate, insulin, wortmannin and PD98059 on VSa13 ECM mineralization. VSa13 cells were seeded in 24-well plates at 2×10^4 cells/well, grown until confluence then treated for mineralization. Mineralizing cultures were then left untreated or treated with IGF-1, insulin, vanadate, PD98059, or wortmannin, alone or in combinations. Mineral deposition was revealed after 4 weeks by von Kossa staining and evaluated by densitometry analysis. Mineralization data are presented as the ratio between individual values and their corresponding controls (non-treated, treated with wortmannin and treated with PD98059). Asterisk indicates values statistically different from corresponding controls ($n \geqslant 3$; $P < 0.05$; one-way ANOVA).

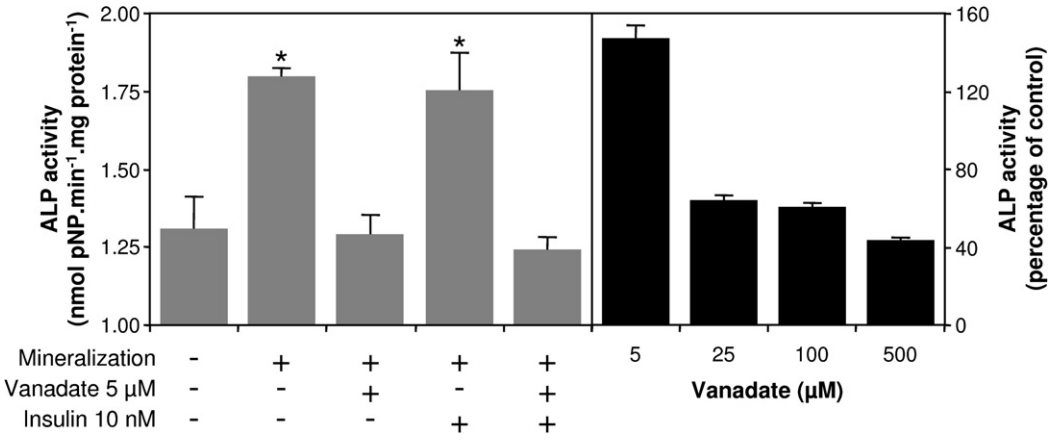

Fig. 3. ALP activity in mineralized cell cultures treated with insulin and vanadate. VSa13 cells were seeded in 24-well plates at 2×10^4 cells/well, grown until confluence then treated for mineralization. Mineralizing cultures were then left untreated (control) or treated with insulin and/or vanadate. ALP activity was measured in cell extracts after 4 weeks of treatment (left panel) or assayed in the presence of increasing concentration of vanadate (right panel; values are normalized with ALP activity measured in the absence of vanadate). Asterisk indicates values statistically different from corresponding controls ($n \geqslant 3$; $P < 0.05$; one-way ANOVA).

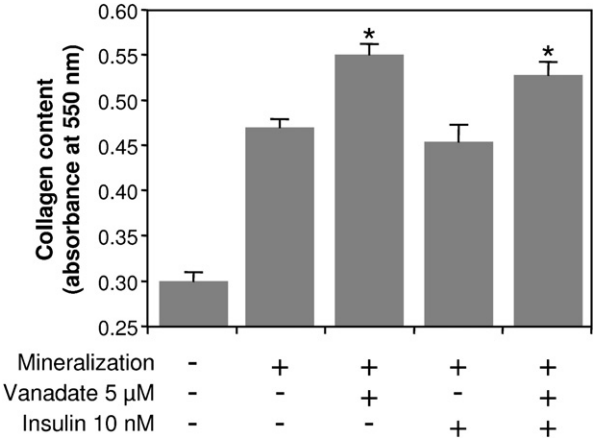

Fig. 4. Collagen content in mineralized cell cultures treated with insulin and/or vanadate. VSa13 cells were seeded in 24-well plates at 2×10^4 cells/well, grown until confluence then treated for mineralization. Mineralizing cultures were then left untreated (control) or treated with insulin and/or vanadate. Total collagen content was determined after 4 weeks of treatment through Sirius red staining. Asterisk indicates values statistically different from corresponding controls ($n \geqslant 3$; $P < 0.05$; one-way ANOVA).

agreement with recent result demonstrating the accumulation of vanadium in VSa13 cells [8], that although cultures were treated with non-inhibitory concentrations of vanadate, these may accumulate intracellularly and reach concentrations inhibitory for ALP activity therefore affecting mechanisms of ECM mineralization. On the contrary, the increase of total collagen content in vanadate-treated mineralized cultures (most probably the result of increased type X collagen as observed in differentiating ATDC5 chondrocyte cells [16]) can hardly explain the decrease of ECM mineralization since increased collagen production is rather likely to benefit ECM mineralization. Similar results (i.e. inhibition of ALP activity and/or stimulation of collagen synthesis) have been reported in chicken [7] and mouse [6,12,20] bone-derived systems, suggesting once again a conservation of mechanisms throughout evolution and across bone cell types.

4. Conclusions

Various evidences have been collected during this work towards the involvement of MAPK and PI-3K/Ras/Erk path-

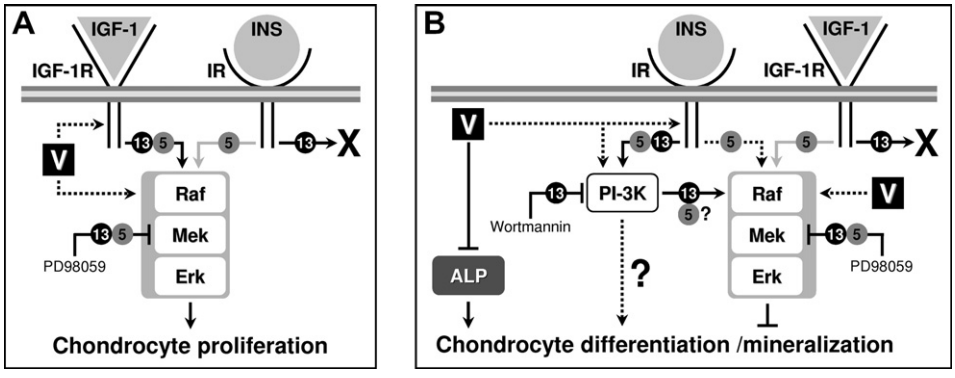

Fig. 5. Putative mechanisms of action for insulin, IGF-1 and vanadate in vertebrate chondrocyte cells. Circled 5 and 13 indicate pathways related to ATDC5 and VSa13 cell lines, respectively. Black, gray and dashed arrows indicate activated, moderately activated and putatively activated pathways, respectively. X indicates an absence of effect. V, vanadate. Raf, Mek and Erk are intermediates in the MAPK pathway.

ways in vanadate proliferative and anti-mineralogenic effects, respectively, using a fish bone-derived cell line of the chondrocyte lineage. We propose that vanadate shares with IGF-1 the signalling pathway regulating chondrocyte proliferation, while it shares with insulin the signalling pathway regulating chondrocyte differentiation/mineralization. We also propose that vanadate not only affects ECM mineralization by activating the PI-3K/Ras/Erk pathway but also inhibits the enzymatic activity of ALP. Finally, this work provides strong evidence for the conservation, throughout evolution, of those mechanisms regulating chondrocyte proliferation and differentiation, emphasizing the suitability of fish to investigate vertebrate development, in particular mechanisms related to chondrogenesis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.03.025.

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