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Macrophage activation by a vanadyl–aspirin complex is dependent on L-type calcium channel and the generation of nitric oxide

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Abstract

Bone homeostasis is the result of a tight balance between bone resorption and bone formation where macrophage activation is believed to contribute to bone resorption. We have previously shown that a vanadyl(IV)–aspirin complex (VOAspi) regulates cell proliferation and differentiation of osteoblasts in culture. In this study, we assessed VOAspi and VO effects and their possible mechanism of action on a mouse macrophage cell line RAW 264.7. Both vanadium compounds inhibited cell proliferation in a dose-dependent manner. Nifedipine completely reversed the VOAspi-induced macrophage cytotoxicity, while it could not block the effect of VO. VOAspi also stimulated nitric oxide (NO) production, the oxidation of dihydrorhodamine 123 (DHR-123) and enhanced the expression of both constitutive and inducible isoforms of nitric oxide syntases (NOS). All these effects were abolished by nifedipine. Althogether our finding give evidence that VOAspi-induced macrophage cytotoxicity is dependent on L-type calcium channel and the generation of NO though the induction of eNOS and iNOS. Contrary, the parent compound VO exerted a cytotoxic effect by mechanisms independent of a calcium entry and the NO/NOS activation.
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1. Introduction

Skeletal integrity is the result of a series of events that depends on the homeostasis between bone formation and bone resorption. In certain pathologies, where this equilibrium is altered, macrophage activation is believed to contribute to bone resorption (Schwartz, 2003; Evans, 2002). Under these conditions, macrophages exert deleterious effects on osteoblast metabolism, al-

Abbreviations: DHR-123, dihydrorhodamine 123; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; IFN-γ, interferon γ; LPS, lipopolysacharide; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^{\bullet}$, peroxynitrite; PBS, phosphate buffered saline; PKC, protein kinase C; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; VO, vanadyl(IV); VOAspi, vanadyl(IV)–aspirin complex

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though the exact relationship between these two types of cells is still unknown.

The trace element vanadium, in its biological active forms of vanadate(V) and vanadyl(IV), exerts insulin mimetic effects such as stimulation of glucose uptake, glycogen synthesis and glucose oxidation (Tsuji and Sakurai, 1996; Dai et al., 1994; Shechter and Shisheva, 1993). In addition, certain vanadium derivatives could induce anti-tumoral effects (Evangelou, 2002; Molinuevo et al., 2004). We have previously synthesized and investigated the effects of a vanadyl–aspirin complex (VOAspi) on osteoblast-like cells in culture (Etcheverry et al., 2000). VOAspi, $[VO(Aspirin)ClH_2O]_2$, is a binuclear complex with carboxylate bridges. In the complex, the vanadium atom presents a square pyramidal coordination sphere like in other vanadium(IV) compounds (Etcheverry et al., 2002). The complex inhibited cell proliferation and differentiation, induced tyrosine phosphorylation of proteins and caused oxidative stress in osteoblastic cell lines (Cortizo et al., 2000a; Etcheverry et al., 2002). In addition, anti-tumoral properties of this compound were demonstrated using the UMR106 osteosarcoma line and a drug delivery system based in a poly(beta-propiolactone) film (Cortizo et al., 2001). We have previously shown that vanadate as well as other vanadium compounds such as pervanadate, vanadyl and VOAspi-induced osteoblastic toxicity generating reactive nitrogen and oxygen species (Cortizo et al., 2000a,b).

In order to further investigate the bioactivity of the VOAspi complex in bone related cells, we assessed its effects and its possible mechanism of action on a mouse macrophage cell line RAW 264.7. The aim of the present study was to asses if VOAspi causes macrophage activation by inducing NO release and increasing the nitric oxide synthase levels. The L-type calcium channel involvement was also investigated.

2. Materials and methods

2.1. Cell culture and incubations

Murine macrophages RAW 264.7 were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 5% (v/v) fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in humidified atmosphere of 95% air and 5% CO_2. For experiments, cells were grown in proper culture-plates. When cells reached 80% confluence, the monolayers were washed twice with serum-free media and cells were incubated with VO or VOAspi at different doses in phenol red-free DMEM during different periods of time. In addition, different agents (nifedipine, a calcium channel blocker; A23187, a calcium ionophore and/or ClK plasma membrane-depolarizing agent) were used to investigate the mechanism of cytotoxicity (Taniguchi et al., 1999; Sozzani et al., 1993).

2.2. Cell proliferation assay

A mitogenic bioassay was carried out as described by Okajima et al. (1992) with some modifications. Briefly, cells in 48 well-plates were washed with phosphate buffered saline (PBS) and fixed with 5% glutaraldehyde/PBS for 10 min. The cells were then stained with 0.5% crystal violet/25% methanol for 10 min. The dye was discarded and the plate was washed with water and dried. The dye taken by the cells was extracted using 0.5 ml/well 0.1 M glycine/HCl buffer, pH 3.0/30% methanol, transferred to test tubes and the absorbance was read at 540 nm. The correlation between the cell number and the absorbance at 540 nm has been previously established (Salice et al., 1999).

2.3. Nitric oxide production

NO production was assessed by measuring the stable end product of NO and nitrite in the culture medium using the Griess' reaction (Cortizo et al., 2000b), using sulfanilic acid as the diazotization agent and *N*-1-napthylethylene diamine as the coupling agent. Briefly, 400 μl samples of conditioned media or nitrite standards 0–100 nM were mixed with 400 μl of Griess' reagent (1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% phosphoric acid) and absorbance was measured at 530 nm against a blank prepared with non-conditioned medium.

2.4. Fluorescence microscopy for nitrogen and oxygen reactive species

Detection of intracellular nitrogen and oxygen species were assessed by the dihydrorhodamine 123 (DHR-123) assay. DHR-123 is no fluorescent and when

it enters cells it is oxidized to the fluorescent rhodamine 123, mainly by peroxynitrite (ONOO•) and •OH. Fluorescent rodhamine 123 is entrapped in the mitochondria (Kooy et al., 1994). This assay was performed with RAW 264.7 macrophages on glass coverslips, which were incubated in DMEM alone or with 100 μM VO or 100 μM VOAspi at 37 °C for 16 h. After that, cell monolayers were washed twice with HBSS and incubated for 1 h with 5 μM DHR. Finally, cells were washed with PBS, fixed and intracellular fluorescence was observed in a Nikon fluorescence microscope.

2.5. Western blot analysis of NO synthases

Cell monolayers were lysated with Laemmli's (1970) Laemmli, 1970 buffer and the protein content in the cell lysates was evaluated by the method of Lowry et al. (1951). The lysate was heated at 100 °C for 3 min and 40 μg of protein was subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were then transferred to PVDF membranes (Millipore, Bedford, MD). To control loading and transfer, the membranes were reversibly stained with 0.2% Ponceau S in 3% trichloroacetic acid before blocking (Cortizo et al., 2000b). After washing with water, the membranes were blocked in 3% non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. Then, they were incubated with anti-iNOS or eNOS polyclonal antibodies (Research Biochemicals International, RBI, MA, USA) in 0.5% bovine serum albumin in PBS (1:2000) at 4 °C for 24 h. After four washes with PBS, 0.1% Tween 20, the membranes were incubated with a secondary goat anti-rabbit antibody, followed by staining with the peroxidase-biotin reagent and diaminobenzidine from the Vectastain kit. The intensity of the iNOS or eNOS specific bands was quantified by densitometry after scanning of PVDF membrane using a Hewlett-Packard Scanjet 4C and the images were analysed using the Scion-beta 2 program.

2.6. Statistical analysis

Student's *t*-test was used for comparisons between basal and experimental groups. All results are expressed as mean ± S.E.M. and represent at least three different experiments.

3. Results

3.1. Vanadium compounds induce macrophage cytotoxicity

We first evaluated the effect of VOAspi on macrophages, measuring RAW 264.7 cell proliferation by the crystal violet bioassay. The parent compound VO was also included for comparison reasons. Fig. 1 shows that both VO and VOAspi significantly inhibited macrophage growth in a dose-dependent manner after 24 h incubation time. The lower doses that significantly inhibited macrophage growth was 100 μM for both VO (85 ± 1% of basal, $p < 0.001$) and VOAspi (60 ± 7% of basal, $p < 0.001$). However, VOAspi was more potent than VO (18 ± 3%, versus 53 ± 8% of basal at 500 μM, respectively). The concentrations of vanadium which inhibited 50% cell growth were: VO ≅ 500 μM and VOAspi = 200 μM, indicating that the effectiveness of VOAspi was higher than that of vanadyl.

The possible mechanism of this cytotoxic effect was further investigated using different agents. As can be seen in Fig. 2, the inhibitory effect caused by 100 μM VO was significantly potentiated by 0.5 μM of the calcium ionophore A23187 ($p < 0.02$) and by

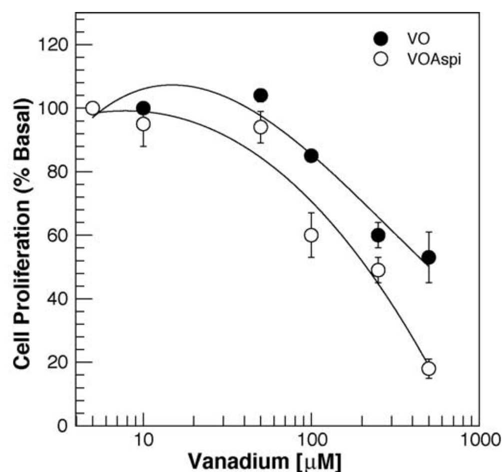

Fig. 1. Effect of vanadium compounds on Raw 264.7 proliferation. Cells were incubated in medium with different doses of VO or VOAspi at 37 °C for 24 h. Cell proliferation was evaluated by the crystal violet bioassay and is expressed as % basal (without vanadium). Values are expressed as mean ± S.E.M. ($n = 6$). Statistically significant differences between basal conditions and treatment with VO or VOAspi are $p < 0.01$.

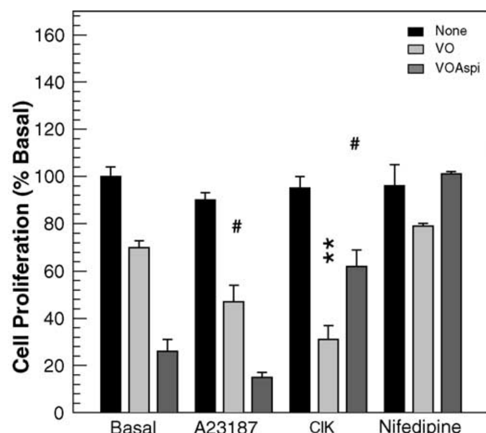

Fig. 2. Effect of different agents on the vanadium-modulated macrophage proliferation. Mouse macrophages were incubated in media alone (basal condition), or with 100 μM vanadyl (VO) or VOAspi at 37 °C for 24 h. Cell proliferation was estimated by crystal violet bioassay. Values are expressed as % basal and represent the mean ± S.E.M. ($n = 3$). Statistically significant differences between the treatment with VO or VOAspi alone and the same treatment plus drugs (0.5 μM A23187, 40 mM KCl or 10 μM nifedipine) are: $^{*}p < 0.001$; $^{**}p < 0.01$; $^{\#}p < 0.02$; $^{x}p < 0.05$.

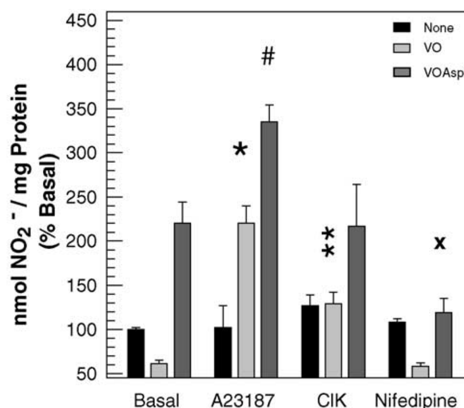

Fig. 3. Effect of different agents on the NO production by macrophages. RAW cells were incubated with media alone (basal condition), 100 μM VO or VOAspi with or without the agents shown in the figure at 37 °C for 24 h. NO production was assessed by Griess' reaction. Values are expressed as % basal and represent the mean ± S.E.M. ($n = 3$). Statistically significant differences between the treatment with VO or VOAspi alone and the same treatment plus drugs (0.5 μM A23187, 40 mM KCl or 10 μM nifedipine) are: $^{*}p < 0.001$; $^{**}p < 0.01$; $^{\#}p < 0.02$; $^{x}p < 0.05$.

40 mM ClK ($p < 0.01$), a plasmatic membrane depolarising agent. The VOAspi-induced cell cytotoxicity at 100 μM was not affected by the addition of A23187, but it was partially reversed by 40 mM KCl ($p < 0.01$) (Fig. 2). Besides, the treatment with the L-type calcium channel blocker, nifedipine, completely reversed the VOAspi-induced macrophage cytotoxicity ($p < 0.001$), while it could not block the effect of VO (Fig. 2).

3.2. Nitric oxide production by macrophages

We next investigated the effect of both vanadium compounds on NO production by mouse macrophages. In Fig. 3 it can be observed that VOAspi causes 2.20-fold increase in NO production ($p < 0.01$, difference versus basal) while vanadyl cation suppressed it (40% under basal, $p < 0.001$). The VOAspi stimulated NO production was significantly potentiated by 0.5 μM A23187 ($p < 0.02$), while KCl did not affect the NO formation. In addition, 10 μM nifedipine completely reversed the VOAspi-stimulated NO production ($p < 0.05$). On the other hand, the 100 μM VO-inhibited NO production was reversed by treatment with 0.5 μM A23187 ($p < 0.001$) or 40 mM ClK ($p < 0.01$), but it was not affected by the addition of nifedipine.

3.3. Intracellular DHR oxidation

Macrophages were assayed for DHR oxidation in culture. Cells incubated in media alone or with 100 μM VO showed a punctuate distribution of weak intracellular fluorescence (Fig. 4a and b). When Raw 264.7 cells were cultured in the presence of 100 μM VOAspi, an increase in the intracellular fluorescence over basal condition was observed (Fig. 4c). Since fluorescent DHR oxidation product is cationic, it is sequestrated in organelles with negative membrane potential. Fluorescence observed in VOAspi-treated cells seems to be associated with the mitochondria. In addition, we assessed the possible effect of nifedipine on the VOAspi-induced DHR oxidation. As can be see in Fig. 4d, 10 μM nifedipine blunted out the intracellular fluorescence.

3.4. Effect of vanadium on endothelial and inducible NO synthases protein expression

We performed western blot analysis to further evaluate the effect of VO and VOAspi on the expression

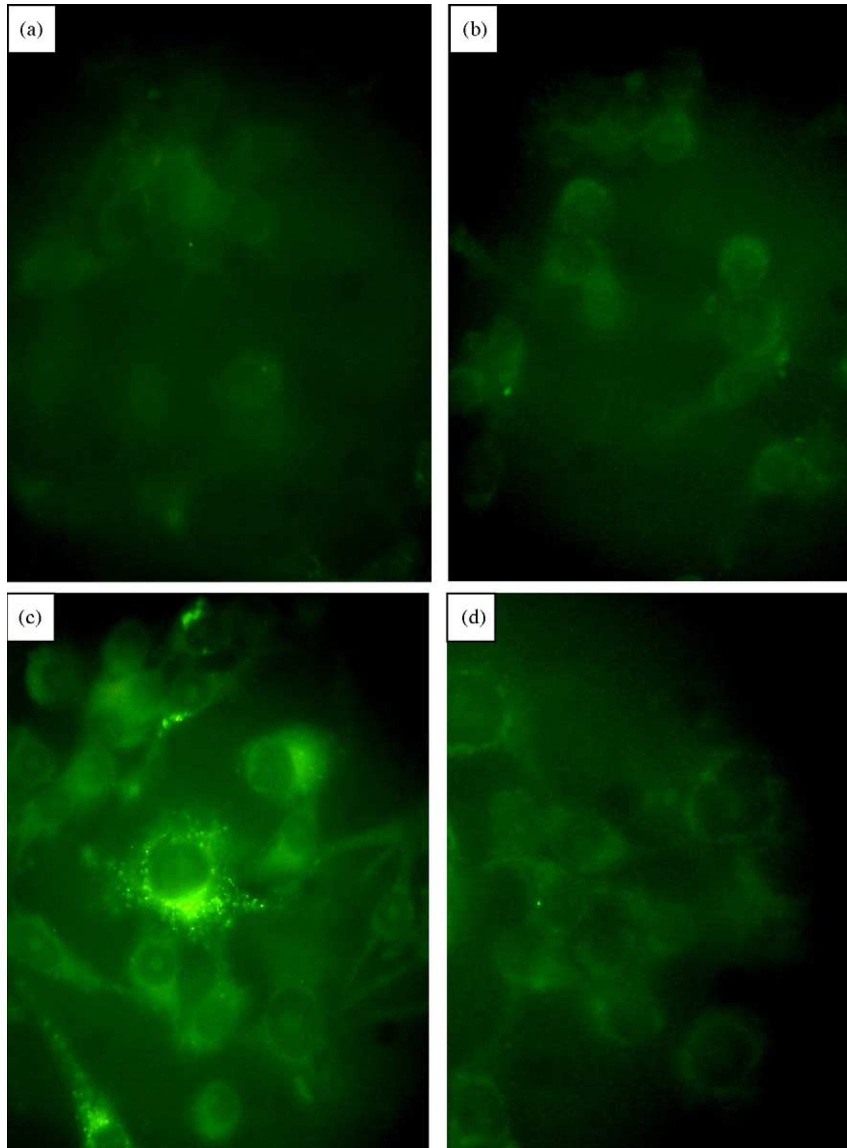

Fig. 4. Fluorescence microscopy of ROS-induced by VOAspi. Macrophages RAW 264.7 was incubated during 24 h at 37 °C in media alone (basal condition) (a) or with 100 μM VO (b), 100 μM VOAspi (c) or 100 μM VOAspi plus 10 μM nifedipine (d). The figures are representatives of three independent experiments. Objective 40×.

of the two isoforms of NOS present in macrophages. Western blot analysis with anti-inducible NOS antibody revealed a band corresponding to a relative mass of 130 kDa, whose expression was enhanced by 100 μM VOAspi (134 ± 8% basal, $p < 0.05$) but not by VO cation (Fig. 5). The induction of iNOS by VOAspi was reverted by nifedipine.

In addition, by using an anti-endothelial NOS antibody, we detected a protein of 135 kDa. VOAspi stimulated the expression of eNOS (142 ± 7%, $p < 0.001$) (Fig. 6), an effect that was partially inhibited by 10 μM nifedipine (118 ± 6%, $p < 0.05$ versus basal). On the other hand, a slight decrease in eNOS expression was detected by the incubation of the macrophages with

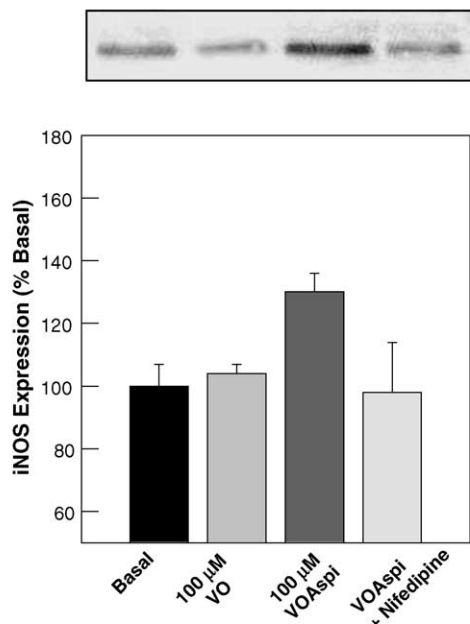

Fig. 5. Determination of iNOS expression on treated RAW 264.7 cells. Western blot analysis of whole cell lisates were performed after incubation of macrophages during 24 h at 37 °C with the drugs as indicated in the figure, a basal condition of non-treated cells was also included. Values are expressed as % basal of iNOS expression and represent the mean ± S.E.M. ($n = 3$).

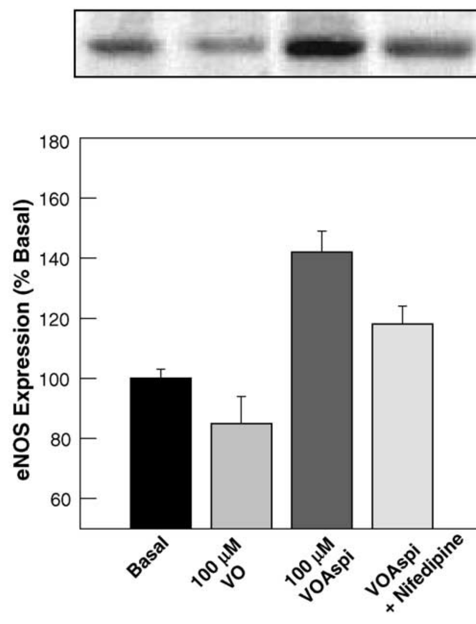

Fig. 6. Study of eNOS expression on treated RAW 264.7 cells. Western blot analysis of whole cell lisates were performed after incubation of macrophages during 24 h at 37 °C with the drugs as indicated in the figure, a basal condition of non-treated cells was also included. Values are expressed as % basal of eNOS expression and represent the mean ± S.E.M. ($n = 3$).

100 μM VO. These results suggest that the VOAspi-induced NO production is mediated by both the increase in eNOS as well as iNOS expression.

4. Discussion

Several vanadium compounds have been proposed for the treatment of diabetes considering its insulin-mimetic properties (Thompson and Orvig, 2004). In addition, anti-tumorigenic actions of vanadium compounds in different in vivo or in vitro models were also demonstrated (Djordjevic, 1995). Before these compounds can be used in vivo, the side effects or the cytotoxicity should be evaluated (Domingo, 2002). In this regard we have previously shown that vanadate, vanadyl and different vanadium(IV) derivatives-induced osteoblastic toxicity trough mechanism involving oxygen and nitrogen reactive species (Cortizo et al., 2000a,b). In the context of skeletal tissue, where vanadium is stored in vivo, additional effects could be exerted on different bone-related cells such as macrophages and/or osteoclasts.

In this study, we have shown that VOAspi and its parent compound VO result cytotoxic to macrophages at concentrations that are similar to the toxic doses for osteoblastic lines (Etcheverry et al., 2000; Salice et al., 1999). VOAspi was also shown to be more toxic (about 2.5-fold) that VO in the present and previous studies in osteoblasts. These concentrations are higher than the range of serum levels attainable in studies of rat-treated with 50 mg/day of vandyl sulphate (10–15 μM) (Shechter, 1990; Thompson et al., 1998). However, when vanadium is accumulated in bone, it could reach considerable higher concentrations (26.4 μg/g wet weight) (Yuen et al., 1993). Thus, the cytotoxic doses found in the present study (>100 μM) are consistent with a dose range attainable in vivo in the microenvironment of the bone.

It was early shown that vanadate and vanadyl interact and inhibit ion-translocating ATPases such as (Na^+, K^+) and (Ca^{2+}, Mg^{2+}). These compounds are

also potent inhibitors of protein tyrosine phosphatases (Nechay, 1984). Subsequently, different key second messengers could be altered by the presence of vanadium, modulating important cell events. The possible involvement of calcium in the VO and VOAspi-induced macrophage cytotoxicity was investigated by using different agents. The inhibitory effect of VO on macrophages growth was enhanced by increasing calcium influx either with the ionophore A23187 or by depolarizing the membrane with ClK, which stimulates Ca^{2+} entry and was not abolished by the L-type calcium channel blocker nifedipine. These observations suggest that calcium entry could be an indirect mechanism by which VO induces macrophage cytotoxicity. On the contrary, VOAspi affects macrophage growth in a manner dependent of a L-type calcium channel activity, since its effect was reverted by membrane depolarization or by adding nifedipine. These data suggest that the calcium influx mediated by an L-type calcium channel could be involved in the VOAspi-induced cytotoxicity in RAW cells.

It is known that nitric oxide markedly affects intracellular calcium homeostasis by influencing the Ca^{2+} release from the intracellular stores and its influx through membrane channels (Clementi, 1998). NO has been reported to influence the activity of several ion channels, including Cav1 (L-type) (Grassi et al., 2004). In order to investigate the role of NO production in the vanadium effects on macrophages, we assessed the levels of NO in the medium of basal and vanadium-treated cells. We showed that VOAspi-induced a significantly increase in the NO production, an effect that was suppressed by nifedipine and potentiated by the calcium ionophore A23187. This effect was in parallel with the oxidation of the peroxynitrite probe DHR-123, indicating the potential for local increases in the formation of peroxynitrite in VOAspi-treated cells. In agreement with the VOAspi-dependent NO production, incubation of macrophages with this vanadium derivative and nifedipine suppressed the intracellular fluorescence of DHR-123 oxidation product. A different mechanism of action was observed for the parent compound vanadyl. It was shown that VO significantly inhibited NO production. This effect has been previously described by other authors (Tsuji and Sakurai, 1996), although their experiments were performed on IFN-γ/LPS-activated macrophages. In that case, the inhibition of NO production was probably exerted on the inducible NOS. It has been recently shown that the VO inhibition of NO production in pulmonary arterial rings was mediated by the PKC-dependent threonine phosphorylation of eNOS (Li et al., 2004). However, the molecular mechanism by which VO exerts these effects remind unclear. In the present study, we showed that the inhibition of NO formation by vanadyl is reverted by the A23187 ionophore, and membrane depolarisation by KCl, but not by nifedipine. On the other hand, no increment of DHR-123 oxidation could be detected in cells treated with VO, suggesting that this metal ion did not induced the formation of peroxynitrite.

The production of NO is dependent on two enzymes present in macrophages, the endothelial and the inducible NOS. In order to investigate the effect of vanadium on the expression of both enzymes, macrophages were treated with VO or VOAspi. We observed that although VO did not affect the expression of neither eNOS nor iNOS, in VOAspi-treated macrophages, a statistically significant enhancement of both NOS were detected by Western blot. These effects were suppressed by the co-incubation of VOAspi and nifedipine, suggesting the involvement of a L-type calcium channel in the mechanism by which this vanadium derivative-induced eNOS and iNOS expression.

In conclusion, altogether our findings give evidence that the VOAspi induces macrophage cytotoxicity through a L-type calcium channel and the generation of NO by enhancing the expression of eNOS and iNOS. Contrary, the parent compound VO exerts a cytotoxic effect by mechanisms independent of NO/NOS activity.

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References

Clementi, E., 1998. Role of nitric oxide and its intracellular signalling pathways in the control of Ca^{2+} homeostasis. Biochem. Pharmacol. 55, 713–718.

Cortizo, A.M., Bruzzone, L., Molinuevo, S., Etcheverry, S.B., 2000a. A possible role of oxidative stress in the vanadium-induced cytotoxicity in MC3T3E1 osteoblast and UMR106 osteosarcoma cell lines. Toxicology 147, 89–99.

Cortizo, A.M., Caporossi, M., Lettieri, G., Etcheverry, S.B., 2000b. Vanadate-induced nitric oxide production: role in osteoblast growth and differentiation. Eur. J. Pharmacol. 400, 279–285.

Cortizo, M.S., Alessandrini, J.L., Etcheverry, S.B., Cortizo, A.M., 2001. A vanadium–aspirin complex controlled release using a poly(beta-propiolactone) film: effects on osteosarcoma cells. J. Biomater. Sci. Polym. Ed. 12, 945–959.

Dai, S., Thompson, K.H., McNeill, J.H., 1994. One-year treatment of streptozotocin-induced diabetic rats with vanadyl sulphate. Pharmacol. Toxicol. 74, 101–109.

Djordjevic, C., 1995. Anti-tumor activity of vanadium compounds. Met. Ions. Biol. Syst. 31, 595–616.

Domingo, J.L., 2002. Vanadium and tungsten derivatives as antidiabetic agents: a review of their toxic effects. Biol. Trace Elem. Res. 88, 97–112.

Etcheverry, S.B., Williams, P.A., Barrio, D.A., Salice, V.C., Ferrer, E.G., Cortizo, A.M., 2000. Synthesis, characterization and bioactivity of a new VO^{2+}–aspirin complex. J. Inorg. Biochem. 80, 169–171.

Etcheverry, S.B., Williams, P.A., Salice, V.C., Barrio, D.A., Ferrer, E.G., Cortizo, A.M., 2002. Biochemical properties and mechanism of action of a vanadyl(IV)–aspirin complex on bone cell lines in culture. BioMetals 15, 37–49.

Evangelou, A.M., 2002. Vanadium in cancer treatment. Crit. Rev. Oncol. Hematol. 42, 249–265.

Evans, C.E., 2002. Bisphosphonates modulate the effect of macrophage-like cells on osteoblast. Int. J. Biochem. Cell Biol. 34, 554–563.

Grassi, C., D'Ascenzo, M., Azzena, G.B., 2004. Modulation of Ca(v)1 and Ca(v)2.2 channels induced by nitric oxide via cGMP-dependent protein kinase. Neurochem. Int. 45, 885–893.

Kooy, N.W., Royall, J., Ischiropoulis, H., Beckman, J., 1994. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Rad. Biol. Med. 16, 149–156.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Li, Z., Carter, J.D., Dailey, L.A., Huang, Y.C., 2004. Vanadyl sulfate inhibits NO production via threonine phosphorylation of eNOS. Environ. Health Perspect. 112, 201–206.

Lowry, O.H., Rosenbrough, N.J., Farr, A.J., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Molinuevo, M.S., Barrio, D.A., Cortizo, A.M., Etcheverry, S.B., 2004. Antitumoral properties of two new vanadyl(IV) complexes in osteoblasts in culture: role of apoptosis and oxidative stress. Cancer Chemother. Pharmacol. 53, 163–172.

Nechay, B.R., 1984. Mechanism of action of vanadium. Ann. Rev. Pharmacol. Toxicol. 24, 501–524.

Okajima, T., Nakamura, K., Zhang, H., Ling, N., Tanabe, T., Yasuda, T., Rosenfeld, R.G., 1992. Sensitive colorimetric bioassays for insulin-like growth factor (IGF) stimulation of cell proliferation and glucose consumption: use in studies of IGF analogs. Endocrinology 130, 2201–2212.

Salice, V.C., Cortizo, A.M., Gomez Dumm, C.L., Etcheverry, S.B., 1999. Tyrosine phosphorylation and morphological transformation induced by four vanadium compounds on MC3T3E1 cells. Mol. Cell. Biochem. 198, 119–128.

Schwartz, A.V., 2003. Diabetes mellitus: does it affect bone? Calcif Tissue Int. 73, 515–519.

Shechter, Y., 1990. Insulin-mimetic effects of vanadate: possible implications for future treatment of diabetes. Diabetes 39, 1–5.

Shechter, Y., Shisheva, A., 1993. Vanadium salts and the future treatment of diabetes. Endeavour 17, 27–31.

Sozzani, S., Molino, M., Locati, M., Luini, W., Cerletti, C., Vecchi, A., Mantovani, A., 1993. Receptor-activated calcium influx in human monocytes exposed to monocyte chemotactic protein-1 and related cytokines. J. Immunol. 150, 1544–1553.

Taniguchi, H., Tanaka, Y., Hirano, H., Tanaka, H., Shigenobu, K., 1999. Evidence for a contribution of store-operated Ca^{2+} channels to NO-mediated endothelium-dependent relaxation of guinea-pig aorta in response to a Ca^{2+} ionophore, A23187. Naunyn Schmiedebergs Arch. Pharmacol. 360, 69–79.

Thompson, K.H., Yuen, V.G., Mc Neill, J.H., Orvig, C., 1998. Chemical and pharmacological studies of a new class of antidiabetic vanadium complexes. In: Tracey, A.S., Crans, D.C. (Eds.), Vanadium Compounds. Chemistry, Biochemistry, and Therapeutic Application. American Chemical Society, Washington, DC, pp. 329–343.

Thompson, K.H., Orvig, C., 2004. Vanadium compounds in the treatment of diabetes. Met. Ions. Biol. Syst. 41, 221–252.

Tsuji, A., Sakurai, H., 1996. Vanadyl ion suppresses nitric oxide production from peritoneal macrophages of streptozotocin-induced diabetic mice. Biochem. Biophys. Res. Commun. 226, 506–511.

Yuen, V.G., Orvig, C., McNeill, J.H., 1993. Glucose-lowering effects of a new organic vanadium complex, bis(maltolato) oxovanadium(IV). Can. J. Physiol. Pharmacol. 71, 263–269.