TIME AND DOSE-RESPONSE STUDY OF THE EFFECTS OF VANADATE ON HUMAN SKIN FIBROBLASTS

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Abstract. Occupational and environmental exposure to vanadium dusts is an increasing problem because it may cause irritation of the skin and of the respiratory tract. The present study was performed to determine the effects of sodium orthovanadate on human skin fibroblasts. The cells treated with vanadate showed clear morphological changes. Our results suggest that vanadate has different effects exposure level-dependent. Low concentrations of vanadate have a mitogenic effect, in a maximum extent at 100 μM, acting as growth factor like insulin. In contrast, in high concentration vanadate caused significant decreases in cell viability and proliferation suggesting that cytotoxicity is due to reactive oxygen species formed.

Key words: human skin fibroblast, vanadate, oxidative stress.

INTRODUCTION

Vanadium is the first element of the fifth group in the periodic system of elements existing in oxidation states ranging from –1 to + 5, preferentially +3, +4 (vanadyl) and +5 (vanadate). Below pH 3.5, vanadium mainly exists as vanadyl (VO^{2+}), while in alkaline solutions the ortho-vanadate form is predominant, which is chemically similar to phosphate. In neutral solutions, vanadium occurs in its pentavalent form as $H_2VO_4^-$, which is the most toxic, as it easily enters the cell, where it is reduced to vanadyl (+4). Vanadyl interacts with cellular molecules, and an involvement in free radical generating processes has been postulated [1].

For people, food is the major source of vanadium exposure, seafood containing higher concentrations of vanadium. High concentrations of 1-8 ppm vanadium are detected in tobacco smoke [2]. Occupational exposure to vanadium is common in oil-fired boiler electricity generating plants and petrochemical, steel and mining industries and it can be found in groundwater, rocks, soils, coal and oil deposits production [11]. Epidemiological studies have indicated a correlation between exposure to airborne vanadium particles and the incidence of cancer in residents of metropolitan areas [15].

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In medicine, vanadium compounds have been administered for the treatment of diabetes, owing to their insulin mimetic effects. Actually, clinical trials are in progress in order to assess the therapeutic application of vanadium in the treatment of types 1 and 2 diabetic patients [3, 10, 13], although some toxic effects after the chronic use of vanadium compounds have also been reported [8].

The biological actions of vanadium compounds are not restricted to their insulin-mimetic properties. Vanadium regulates growth factor-mediated signal transduction pathways, promotes cell transformation, decreases cell adhesion, etc. [18]. Two kinds of vanadium effects have been described in cultured cells: metabolic or short time actions and mitogenic or chronic effects. The bioactivity of different vanadium compounds has been tested on several cell types. In Swiss 3T3 fibroblasts it has been demonstrated that vanadium induced cell proliferation and cytotoxic effects, in a manner depending on the dose and chemical structure of the compound [4]. Using a model of osteoblast-like cells in culture, Cortizo *et al.* [5–7] suggested that oxidative stress is involved in vanadium induced osteoblastic cytotoxicity, although the mechanism is unknown.

The aim of the present study was to obtain a deeper insight into the effects of vanadate on the proliferation, cytotoxic effects and formation of reactive oxygen species (ROS) in human skin fibroblasts.

CELL CULTURE AND *IN VITRO* VANADATE TREATMENT

Human skin fibroblasts were obtained from biopsies taken from two patients undergoing plastic surgery. Fibroblasts were derived from the tissues by explant techniques in 30-mm diameter tissue culture dishes. They were cultured in monolayer using 75 and 25 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM D-glucose, pyruvate, 10 % fetal bovine serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. Fibroblasts were subcultured in a humidified 5% CO_2/air-mix incubator, and were passaged every 7–10 days. Cells of the fifth to eighth generation were used in the study to avoid effects of culture ageing. Informed consent was obtained from each patient, and approval was received from the local committee for research in human subjects in accordance with the Declaration of Helsinki.

Cells were routinely plated at a density of 8,000 per cm^2 in serum-free medium for 24 h before vanadium treatment. After the culture reached 70 % confluence, the cells were washed with PBS and treated with different concentrations of sodium orthovanadate for 4-24 h in serum-free medium

MATERIALS AND METHODS

The morphological changes were examined with an inverted phase-contrast microscope Nikon Eclipse TS 100 at various time points after addition of sodium vanadate in the culture medium.

Viable cell count was performed by the trypan blue exclusion method. After trypsinization, a 100 µl aliquot of the treated cells was transferred to an Eppendorf tube and used for the viability assay. These cells were diluted (1:10) in PBS and stained with an equal volume of a 0.08 % trypan blue solution for 2 minutes. The cells were counted with the use of a Thoma-Zeiss chamber. The percentage of viable cells was calculated as viable cells (unstained)/ total cells (stained plus unstained) × 100.

Cytotoxicity test was performed using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenases to the corresponding blue formazan. Fibroblasts were treated in 24-well plates and incubated with 200 µl/well of MTT (1 mg/ml) in medium for 2-4 h. The reaction was stopped by addition of 200 µl of 10 % (w/v) SDS/0.01 M HCl per well, and the formazan released from the cells by incubation at 37 °C overnight was measured. Absorbance of the supernatant was measured at 570 nm against a background at 700 nm. Cytotoxicity was calculated as the percentage of formazan formation in cells treated with vanadate compared with control cells.

Cell proliferation assay was carried out with crystal violet as described Cortizo and Etcheverry [4]. Briefly, cells in 24-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 which the dye solution was discarded and the plate was washed with acidified (pH 2.5) 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. Cortizo and Etcheverry [4] found o good correlation between the total cell number and the absorbance at 540 after crystal violet staining.

Lipid peroxidation was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) by the method of Esterbauer and Cheeseman [9]. The TBA reagent was prepared by dissolving 0.67% TBA (wt/vol) in 50% acetic acid and adding 0.01% (wt/vol) butylated hydroxytoluene. After vanadate treatment, the cells were washed with PBS and scraped in 0.1 % Triton X-100. To 0.5 ml of cell lysate, 0.5 ml of TBA reagent was added and heated in a boiling water bath for 15 min. After cooling on ice, the fluorescence intensities of samples were finally measured at excitation and emission wavelengths of 535 nm and 553 nm, respectively. Medium without cells was assayed as the blank, exactly under the same conditions. For quantification a standard curve was prepared by using a 100 m*M* 1,1,3,3-tetraethoxypropane stock solution diluted in ethanol, and normalized to the cell protein content. Data were expressed as nmol TBARS per 100 mg of protein.

Detection of intracellular ROS was performed by oxidation of dihydrorhodamine 123 (DHR) to rhodamine (RH) according to Royall and Ischiropoulos [16]. Briefly, the cells were incubated at 37°C in PBS (basal

condition) or with different concentrations of vanadate in the presence of 10 μM DHR. The cell extracts (monolayers rinsed with PBS and scraped in 0.1 % Triton X-100) were analyzed for the RH presence by measuring fluorescence intensities at excitation and emission wavelengths of 495 and 532 nm, respectively. Results corrected for protein content were calculated using a standard curve of RH from 0.1 to 5 μM.

Protein concentration was determined by the method of Shopsis and Mackay [17].

For each experiment, duplicate plates for untreated or treated cultures were assayed. All experiments were performed in duplicate.

RESULTS AND DISCUSSION

In all experiments, we used the fibroblasts derived from the same donor and in the same subculture. Twenty-four hours prior to exposure to vanadate, the growth medium was changed to fresh serum-free DMEM and the fibroblasts were incubated for 24 h at 37 °C in a humidified 5% CO_2/air-mix incubator. Freshly prepared vanadate was added directly to the culture medium at final concentrations of 10, 50 and 100 μM (low doses) and 1 and 10 mM (high doses). To avoid changes in pH, vanadate was added in volumes that were a maximum of 1 % of the culture volume to yield the given final concentrations. The exposition time to vanadate at 37 °C was 4 and 24 h.

Human skin fibroblasts, grown as monolayers, have a stellate shape with slender lamellar expansions in DMEM supplemented with FBS and a polygonal dendritic morphology when they were deprived of serum for 24h. The cells treated with vanadate showed clear morphological changes. Cells treated with low doses of vanadate were very dense, fusiform and smaller in shape, had less processes, with clearly defined borders. Cultures treated with 10 mM vanadate for 24 h showed a very few cells with more condensed cytoplasm and long processes, many free-floating and dead cells (data not shown).

Table 1

Effects of different concentrations of vanadate on the viability, cytotoxicity and proliferation of human skin fibroblasts (treatments for 4 and 24 h at 37°C). Data are expressed as % over basal

Vanadate	% viable cells		Cytotoxicity		Proliferation	
	4 h	24 h	4 h	24 h	4 h	24 h
0	100	100	0	0	100	100
10 μM	96±2.9	98± 2.4	106±12.4	26±2.5	102±6.1	107±3.3
50 μM	99±2.2	99± 1.3	101±19.2	31±4.7	114±3.5	111±2.4
100 μM	93±5.5	95± 1.8	101±7.8	48±6.5	121±2.3	136±3.0
1 mM	87±4.7	72± 7.2	91±9.4	68±11.3	109±2.1	114±8.1
10 mM	49±9.1	58±10.8	88±6.1	95±8.4	46±7.7	26±4.6

Table 1 shows the effect of different vanadate concentrations on viability, cytotoxicity and proliferation of human skin fibroblasts incubated for 4 and 24 h. The treatment achieved with vanadate shows different responses with the dose and with the application time. At all time points examined, exposure to low doses of vanadate had no effect on cell viability. In contrast, exposure of cells to 1 or 10 mM Na_3VO_4 caused significant decreases in cell viability, more evident at higher dose and after 48 h of treatment. A dose-dependent increase in cytotoxicity was observed after 24 h of exposure to vanadate at all concentrations. At 4 h of exposure, only low doses of vanadate did not show a cytotoxic effect, concentrations of 1 or 10 μM Na_3VO_4 induced comparable levels of cytotoxicity.

To determine if the human skin fibroblasts were modulated by vanadate acting as growth factor like insulin, we evaluated its effects on cellular proliferation. Low doses of vanadate stimulated cell proliferation to a maximum extent at 100 μM and 24 h of exposure. The highest dose of vanadate induced a pronounced decrease in cell proliferation in a time-dependent manner.

To assess the biochemical mechanisms of toxicity induced by high doses of vanadate, we studied the lipid peroxidation of vanadate-treated fibroblasts after a 4h and 24h incubation. The measurement of TBARS released in the supernatant of cultured cells can be considered as a reliable and representative index of peroxidation with regard to vanadate-induced oxidative stress.

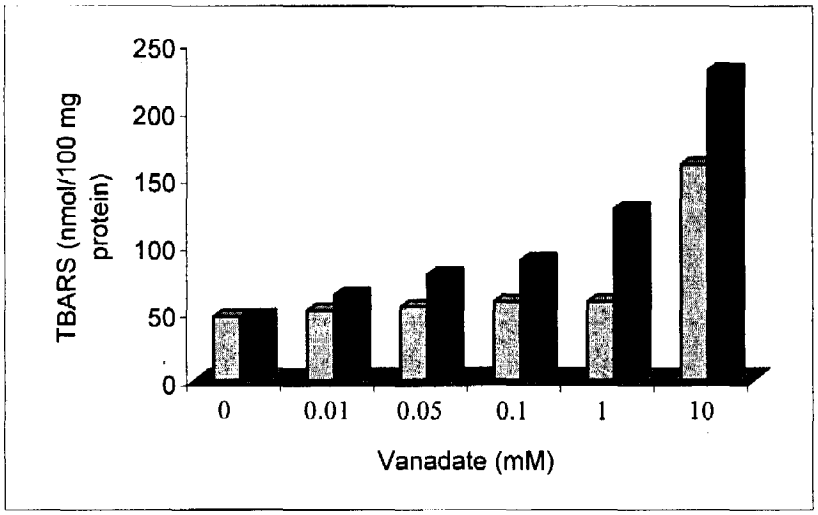

Fig. 1. – Intracellular TBARS production in cells treated with different levels of vanadate (grey – for 4 h, black for 24 h).

At 4 h of exposure, vanadate at doses between 10 μM and 1 mM did not alter the TBARS production, but an increase in lipid peroxidation with 242.6% was assessed at the highest concentration. In the culture medium of human skin

fibroblasts treated with vanadate for 24 h, the level of TBARS increased in a dose-dependent manner suggesting an oxidative stress associated with cellular death or membrane injury that may explain the toxic manifestations of this transition metal (Fig. 1).

To confirm the possible role of oxidative stress in the vanadate-induced cytotoxicity in human skin fibroblasts we studied the ROS formation measured by oxidation of dihydrorhodamine 123 to rhodamine (Fig. 2). The incubation of cells with low doses of vanadate (10 – 100 μM) was not associated with intracellular ROS generation, but concentrations of 1 and 10 mM vanadate induced a dose-dependent increase in the ROS formation, more pronounced after 4 h exposure. Antioxidant mechanisms of defense may be activated at prolonged exposure, suggesting an adaptive response to oxidative stress in fibroblasts incubated for 24 h with 1 and 10 mM vanadate. The level of free radicals inside the cells is a product of a balance between their generation and the antioxidant defenses in the cells.

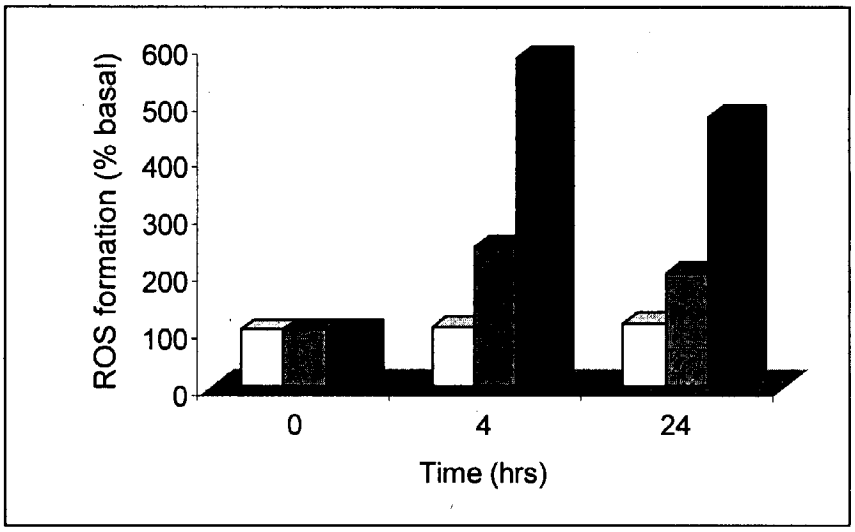

Fig. 2. – ROS formation in human skin fibroblasts treated with vanadate: 0.1 mM (white); 1 mM (grey) and 10 mM (black).

Our studies show that vanadate can cause an oxidative stress through the formation of TBARS and ROS in fibroblasts and suggest that the vanadium-induced cytotoxicity is partially dependent on oxidative stress, in a concentration-dependent manner. Similar effects were described by Cortizo *et al.* [7] in a study concerning the action of vanadium compounds on some osteoblast and osteosarcoma cell lines. Huang *et al.* [14] have demonstrated that exposure of mouse epidermal cells to vanadate induces generation of H_2O_2 that is able to cause p53 activation in a time- and dose-dependent manner and this treatment caused mitochondrial damage and cell apoptosis.

In summary, the results presented in this study demonstrated that vanadate has different effects depending on concentration, at least in human skin fibroblasts. In low concentration, vanadate has a mitogen effect acting as a growth factor like insulin and a possible anti-oxidant role. In contrast, in high concentration vanadate exerts potent toxic effects, but mechanisms controlling its adverse effects remain to be elucidated. In view of occupational exposure, the carcinogenic effects of vanadium compounds and their utilization in medicine for the treatment of diabetes make necessary future investigation.

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REFERENCES

1. ALTAMIRANO-LOZANO, E., E. ROLDAN-REYES, E. ROJAS, *Genetic Toxicology of Vanadium Compounds: Vanadium in the Environment*, Parts I and II, John Wiley & Sons, New York, 1997.
2. BARCELOUX, D. G., Vanadium, *Clin. Toxicol.*, 1999, **37**, 265–278.
3. COHEN, N., M. HALBERSTAM, P. SHLIMOVICH, C. J. CHANG, H. SHAMOON, L. ROSSETTI, Oral vanadyl sulfate improves hepatic and peripheral insulin sensitivity in patient with non-insulin-dependent diabetes mellitus, *J. Clin. Invest.*, 1995, **95**, 2501–2509.
4. CORTIZO, A. M., S. B. ETCHEVERRY, Vanadium derivatives act as growth factor – mimetic compounds upon differentiation and proliferation of osteoblast-like UMR106 cells, *Mol. Cell. Biochem.*, 1995, **145**, 97–102.
5. CORTIZO, A. M., V. C. SALICE, C. M. VESCINA, S. B. ETCHEVERRY, Proliferative and morphological changes induced by vanadium compounds on Swiss 3T3 fibroblasts, *BioMetals*, 1997, **10**, 127–1333.
6. CORTIZO, A. M., S. B. ETCHEVERRY, Vanadate-induced nitric oxide production: role in osteoblast growth and differentiation, *Eur. J. Pharmacol.*, 2000, **400**, 279–285.
7. CORTIZO, A. M., L. BRUZZONE, S. MOLINUEVO, S. B. ETCHEVERRY, A possible role of oxidative stress in the vanadium-induced cytotoxicity in the MC3T3E1 osteoblast and UMR106 osteosarcoma cell lines. *Toxicology*, 2000, **147**, 89–99.
8. DOMINGO, J. L., M. GOMEZ, D. J. DANCHEZ, J. M. LLOBET, C. L. KEEN, Tiron administration minimizes the toxicity of vanadate but not its insulin mimetic properties in diabetic rats, *Life Sci.*, 1992, **50**, 1311–1317.
9. ESTERBAUER, H., K. H. CHEESEMAN, Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol.*, 1990, **186**, 407–421.
10. GOLDFINE, A. B., D. C. SIMONSON, F. FOLLI, M. E. PATTI, C. R. KAHN, Metabolic effects of sodium metavanadate in humans with insulin-dependent and non-insulin-dependent diabetes mellitus *in vivo* and *in vitro* studies, *J. Clin. Endocrinol. Metab.*, 1995, **80**, 3311–3320.
11. GOYER, R. A., Toxic effects of metals. In: *Toxicology*, 4th edn., M. O. Amdur, J. Doull, C. D. Klassen eds., Pergamon Press, New York, 1991, pp. 623–680.
12. GREEN, L. M., J. L. READE, C. F. WARE, Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines, *J. Immunol. Methods*, 1984, **70**, 257–268.
13. HALBERSTAM, M., N. COHEN, P. SHLIMOVICH, L. ROSSETTI, H. SHAMOON, Oral vanadyl sulfate improves insulin sensitivity in NIDDM but not in obese non-diabetic subjects, *Diabetes*, 1966, **45**, 659–666.

14. HUANG, C., Z. ZHANG, M. DING, J. LI, J. YE, S.S. LEONARD, H. M. SHEN, L. BUTTERWORTH, Y. LU, M. COSTA, Y. ROJANASAKUL, V. CASTRANOVA, V. VALLYATHAN, X. SHI, Vanadate Induces p53 Transactivation through Hydrogen Peroxide and Causes Apoptosis. *J. Biol. Chem.*, 2000, **275**, 32516–32522.
15. KRAUS, T., H. RAITHEL, K. H. SCHALLER, Quantitation of manganese and vanadium levels in the lungs of normal and occupationally exposed persons, *Zentralbl. Hyg. Umwelimed.*, 1989, **188**, 108–126.
16. ROYALL, J. A., H. ISCHIROPOULOS, Evaluation of 2',7'-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells, *Arch. Biochem. Biophys.*, 1993, **302**, 348–355.
17. SHOPSIS, C., G. I. MACKAY, Semi automated assay for cell culture, *Anal. Biochem.*, 1984, **140**, 104–107.
18. STERN, A., X. YIN, S. S. TSANG, A. DAVISON, J. MOON, Vanadium as a modulator of cellular regulatory cascades and oncogene expression. *Biochem. Cell Biol.*, 1993, **71**, 103–112.