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Summary

After 24 hours, cardiac myocytes exposure to 10 μM (LD_{50}) vanadate (meta or decavanadate) an increased (30%) of caspase 3-activation was observed, although not significant. On contrary, a significant decrease (40%) of ATP content, characteristic of necrotic cell death was detected. Furthermore, vanadate treatment increased intracellular Ca^{2+} level from 60 nM to 240 nM, whereas it decreases mitochondria superoxide anion generation and induces mitochondria membrane depolarization (IC_{50}=6.5 μM). In conclusion, micromolar vanadate exposure induces large chances in two major bioenergetic markers in cardiac myocytes: intracellular calcium concentration and superoxide anion mitochondrial production, suggesting a necrotic cell death through a mitochondrial toxic pathway.

Introduction

Environmental contamination by toxic metals, in particular by vanadium, has increased dramatically during the last decades due to the use of fossil fuels. In spite of the emerging interest in the pharmacological effects of some vanadium compounds, for instance as an insulin-mimetic, in the treatment of diabetes, the toxicology of vanadium constitutes an area of increasing interest. Recently, our research group has been carrying out studies about the toxic effects of vanadate. *In vivo* studies, shown that the degree of vanadium toxicity depends on the mode of administration, such as intraperitoneal or intravenous, and is also dependent, at some extend, to the chemical form of va-

nadium such as decavanadate (1). It was also shown that vanadium is accumulated in hepatic and cardiac muscle cells, inducing changes in lipid peroxidation and oxidative stress markers. One of the most important results was the observation that vanadate inhibits mitochondrial oxygen consumption and induces membrane depolarization at the nM range of concentration (2). In another line of research, the effects of vanadate on the mineralization of a bone-derived cell line were studied and compared to that of insulin. Vanadate (5 μM) was shown to stimulated growth performance and prevents extracellular matrix mineralization, probably through multiple processes involving regulation that may or may not depend upon the activation of insulin stimulated pathways (3). Other studies included the use of vanadate as a probe in the understanding of muscle contraction, calcium homeostasis and actin filaments dynamics (1). In the present study, we described the mechanisms of cell death induced by vanadate using cardiomyocytes. Apparently, vanadate induces necrotic cardiac cell death through impairment of mitochondrial function. We believe that these recent advances in a better knowledge of vanadium toxicity allow a better understanding of vanadium effects in biology and pharmacology (1).

Materials And Methods

Ventricles from hearts of neonatal (2-days-old) Wistar rats (*Rattus norvegicus*) were used. After 3 days in growth medium (DMEM), the cardiomyocytes were treated with decavanadate (DV) or metavanadate (MV) concentrations ranging from 0-50 μM (total vanadium). Metavanadate stock solution (50 mM, pH 6.7) was prepared from ammonium metavanadate. Decavanadate stock solution was obtained by adjusting the pH of the former solution to 4.0. The composition and stability of the vanadate solutions were analyzed by NMR and UV/Vis spectroscopy as described elsewhere (1). All experiments were performed using cardiomyocytes at 3-4 DIV (days in vitro). The purity of cardiomyocytes cell cultures (~80% of myocytes in culture) was estimated either by cell counting, immunodetection of myosin heavy chain or immunostaining. Estimation of viable cells was assessed by measuring the amount of coloured formazan formed upon the reduction of MTT. Caspase-3 activity was measured using Ac-DEVDpNA. Adenine nucleotides were determined spectrophotometrically after extraction with perchloric acid (3.3%) and neutralization with KOH. Intracellular calcium ($[Ca^{2+}]i$) was measured using the fluorescent indicator fura-2. Dihydroethidium (DHE) was used as a specific dye for superoxide anion. Mitochondrial membrane potential (Ψm) was monitored in cardiomyocytes with JC-1. The ratios of JC-1 aggregate to monomer intensity for each region were calculated. A decrease in this ratio was interpreted as decrease of Ψm, whereas an increase in the ratio was interpreted as again in Ψm (Soares et al, in revision process).

Results And Discussion

Both vanadate solutions were found to decrease cell viability (not shown).

MTT assay indicated that 24 h exposure to decavanadate (DV) and metavanadate (MV) solutions induce a similar loss of cell viability (IC50 = 10 μM total vanadium). No caspase-3 activation was observed after 12 hours exposure to decavanadate or metavanadate concentrations. However, 24 hours after and increase (30%) of caspase activity was detected, although not significant. On contrary, a significant decrease of the cardiomyocytes energy charge was observed after 24 h exposure to DV: 10 and 20 μM total vanadium reduced energy charge by 36% and 39%, respectively, and lower decrease (15%) and (20%) to the same concentrations of metavanadate. Control cardiac myocytes (non treated cells) presented a normal low $[Ca^{2+}]i$ in the range of 60±10 nM. Cardiomyocytes treated for 24 hours with decavanadate or metavanadate (10 μM total vanadium) were able to maintain its intracellular Ca^{2+} homeostasis although at higher basal $[Ca^{2+}]i$, between 200 and 250 nM. Therefore, these results pointed out that cardiomyocytes exposure to vanadate solutions, either decavanadate and metavanadate, impaired the balance between calcium uptake and release by intracellular Ca^{2+} stores, favouring partial calcium depletion of intracellular stores. The intracellular oxidative stress monitored by DHE was found to be largely attenuated by exposure of cardiomyocytes to both vanadate solutions. In fact, a 24 h treatment of cardiomyocytes with 20 μM decavanadate (i.e. 2 μM DV) or metavanadate solutions, reduced anion superoxide production by 70%. Maintenance of the mitochondrial membrane potential (Ym) is essential for the normal performance and survival of cells with a high metabolic energy requirement, such as cardiomyocytes. Mitochondrial membrane potential plays a major role in the modulation of calcium uptake and release by mitochondria in rat ventricular myocytes. Fluorescence images of cardiomyocytes stained with JC-1 showed that control cells exhibited heterogeneous staining of the cytoplasm with both red (polarized mitochondria) and green (depolarized mitochondria) fluorescence co-existing in the same cell (not shown). Consistent with mitochondrial localization, the red fluorescence was mostly found in rod-shaped and granular structures distributed throughout the cytoplasm. The green fluorescence followed a similar pattern but overall gave a more blurred image, suggesting also some extra-mitochondrial localization. Pretreatment of cardiomyocytes with 1 μM decameric vanadate (i.e., 10 μM total vanadium) for 24 hours had a dramatic effect on the red fluorescence, which now became very faint (Soares et al, in revision process). It was verified that 24 h incubation of cardiomyocytes with decavanadate and metavanadate solutions, induces the collapse of Ym, with the same IC50 value of 6.5 μM total vanadium (i.e. 0.65 μM decameric vanadate or 6.5 μM monomeric vanadate). In addition, mitochondria membrane depolarization is likely underlying the observed rise of basal cytosolic calcium and partial depletion of intracellular calcium stores upon incubation of cardiomyocytes with decavanadate or metavanadate solutions, as mitochondria membrane depolarization leads to mitochondrial calcium release and also IP3-mediated endoplasmic reticulum release in cardiomyocytes.

Conclusions

We have found that incubation of cardiomyocytes with either decavanadate or metavanadate solutions for 24 h produced large changes in two major bioenergetic markers in these cells: (i) basal cytosolic Ca^{2+} concentration, and (ii) mitochondrial production of superoxide anion. In this study, we also verified that 24 h incubation of cardiomyocytes with decavanadate and metavanadate solutions induce the collapse of Ym, with the same IC50 value of 6.5 μM. Thus, our results strongly suggest that mitochondrial membrane depolarization is a key event in decavanadate- and metavanadate-induced cardiomyocytes death, which is noticeable after 24 h incubation with concentrations as low as 2 μM total vanadium. In conclusion, exposure to micromolar concentrations of vanadate (added from decavanadate or metavanadate solutions) induces cell death in isolated neonatal rat ventricular myocytes through a similar toxic pathway, leading to mitochondrial membrane depolarization and resulting in necrotic cell death.

References

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