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Vanadate-induced activation of activator protein-1: role of reactive oxygen species

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The present study was undertaken to test the hypothesis that the toxicity and carcinogenicity of vanadium might arise from elevation of reactive oxygen species leading to activation of the transcription factor activator protein-1 (AP-1). The AP-1 transactivation response has been implicated as causal in transformation responses to phorbol esters and growth factors. To investigate the possible activity of vanadium in the activation of AP-1, we treated mouse epidermal JB6 P^+ cells stably transfected with an AP-1 luciferase reporter plasmid with various concentrations of vanadate. This resulted in concentration-dependent transactivation of AP-1. Superoxide dismutase (SOD) and catalase inhibited AP-1 activation induced by vanadate, indicating the involvement of superoxide anion radical ($O_2^{-}\cdot$), hydroxyl radical (·OH) and/or H_2O_2 in the mechanism of vanadate-induced AP-1 activation. However, sodium formate, a specific ·OH scavenger, did not alter vanadate-induced AP-1 activation, suggesting a minimal role for the ·OH radical. NADPH enhanced AP-1 activation by increasing vanadate-mediated generation of $O_2^{-}\cdot$. *N*-acetylcysteine, a thiol-containing antioxidant, decreased activation, further showing that vanadate-induced AP-1 activation involved redox reactions. Calphostin C, a specific inhibitor of protein kinase C (PKC), inhibited activation of AP-1, demonstrating that PKC is involved in the cell signal cascades leading to vanadate-induced AP-1 activation. Electron spin resonance (ESR) measurements show that JB6 P^+ cells are able to reduce vanadate to generate vanadium(IV) in the presence of NADPH. Molecular oxygen was consumed during the vanadate reduction process to generate $O_2^{-}\cdot$ as measured by ESR spin trapping using 5,5-dimethyl-L-pyrroline *N*-oxide as the spin trapping agent. SOD inhibited the ESR spin adduct signal, further demonstrating the generation of $O_2^{-}\cdot$ in the cellular reduction of vanadate. These results provide support for a model in which vanadium, like other classes of tumor promoters, transactivates AP-1-dependent gene expression. In the case of vanadium, AP-1 transactivation is dependent on the generation of $O_2^{-}\cdot$ and H_2O_2, but not ·OH.

Abbreviations: AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; DMPO, 5,5-dimethyl-L-pyrroline *N*-oxide; EMEM, Eagle's minimal essential medium; ESR, electron spin resonance; FBS, fetal bovine serum; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Introduction

Vanadium is a transition metal widely distributed in the environment and in biological systems. Occupational exposure to vanadium is common in oil-fired boiler electricity generating plants and the petrochemical, steel and mining industries and it can be found in groundwater, rocks, soils, coal and oil deposits (1–5). Epidemiological studies have indicated a correlation between exposure to airborne vanadium particles and the incidence of cancer in residents of metropolitan areas (6,7). Vanadium regulates growth factor-mediated signal transduction pathways, promotes cell transformation and decreases cell adhesion (8–10). In the periodic table, vanadium belongs to the first transition series and can form compounds mainly in valencies III, IV and V. The vanadium(III) species are unstable at physiological pH and in the presence of oxygen. Vanadium(IV) is easily oxidized to vanadium(V) under physiological conditions and vanadium(V) species are found as vanadate anions. Vanadate(V) and vanadate-containing compounds exert potent toxic and carcinogenic effects on a wide variety of biological systems. Mutations and DNA–protein crosslinks induced by vanadate in cultured mammalian cells have been reported (11). The transforming activity of vanadate has been studied in BALB/3T3 cells (12). The results indicate that vanadate may act both as an initiator and a promoter of morphological transformation of cultured mammalian cells.

While the biochemical mechanisms of toxicity and carcinogenicity induced by vanadium are still not fully understood, recent studies have indicated that vanadium-mediated generation of reactive oxygen species (ROS) may play an important role in vanadium-induced toxicity (13–16). For example, reduction of vanadate by glutathione reductase in the presence of NAD(P)H generates vanadium(IV) (16). During the reduction process, molecular oxygen is reduced to $O_2^{-}\cdot$ and then to H_2O_2 via dismutation. The reaction of vanadium(IV) with H_2O_2 also generates hydroxyl radical (OH·) via a Fenton-like reaction (14).

In the present investigation, we studied the effect of vanadium-mediated generation of ROS and its possible role in the activation of activator protein-1 (AP-1) activity using mouse epidermal JB6 cells. The mouse JB6 cell model allows precise study of molecular events required in tumor promoter-induced neoplastic transformation. Both $O_2^{-}\cdot$ generation (17,18) and transactivation of the transcription factor AP-1 (19–24) have been definitively shown to be required for the induced transformation response in JB6 cells. Recently JB6 reporter cells (25,26) have been used to identify retinoids, antioxidants and other agents that transrepress AP-1, as well as to identify and characterize transactivators such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), epidermal growth factor and tumor necrosis factor α (TNFα). These reporter cells stably express −76 to +63 of the human (MMP-)collagenase promoter containing a single AP-1 site driving a luciferase reporter, thus circumventing the need for transient transfection and normalization of transfection efficiency.

This study was undertaken because AP-1 appears to play a key role in the induction of several genes associated with transformation, proliferation, differentiation and tumor promotion (19–24,27,28). AP-1 is a transcription factor composed of homodimers and/or heterodimers of the Jun and Fos families. The genes encoding these proteins, *jun* and *fos*, are inducible by a variety of extracellular stimuli and function as intermediary transcription regulators in signal transduction processes leading to proliferation and transformation (19–24). The activity of AP-1 is modulated by several factors, including the redox state of the cells (29–33). A recent report has indicated that vanadium may induce c-*jun* expression (10) an event that is not necessarily limiting for AP-1 activation (34,35). Whether vanadium could cause AP-1 activation and the mechanism of its induction are not clear. The results obtained from the present study show that vanadate is able to cause AP-1 activation in JB6 cells. $O_2^{-}\cdot$ and H_2O_2 but not ·OH are involved in the mechanism of activation.

Materials and methods

Reagents

Sodium vanadate, superoxide dismutase (SOD), NADPH, *N*-acetylcysteine, 5,5-dimethyl-L-pyrroline *N*-oxide (DMPO), sodium formate and H_2O_2 were purchased from Sigma (St Louis, MO). Calphostin C was purchased from LC Laboratory (Woburn, MA). Catalase was purchased from Boehringer Mannheim (Indianapolis, IN). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation. DMPO solution thus purified did not contain any electron spin resonance (ESR)-detectable impurities. Chelex 100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA). The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants.

Cell culture

Stable AP-1 luciferase, NF-κB–chloramphenicol acetyltransferase (CAT) double reporter plasmid-transfected mouse epidermal JB6 P^+ cells, JB6/AP/κB (25,26), were cultured in Eagle's minimal essential medium (EMEM) containing 5% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μg gentamicin/ml. The cells were grown at 37°C in a 5% CO_2 atmosphere. Maintenance of the transgene was ensured by periodic growth in medium containing G418 and hygromycin. For the experiments reported herein, only the AP-1 luciferase reporter was utilized.

Assay for AP-1 activity

Confluent monolayer of JB6/AP/κB cells were trypsinized and 5×10^4 viable cells suspended in 1 ml of 5% fetal bovine serum (FBS) EMEM were added to each well of a 24-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. Twelve to twenty-four hours later, cells were cultured in 0.5% FBS EMEM for 12–24 h to minimize basal AP-1 activity and then exposed to various reagents for 24 h for AP-1 induction in the same medium. The cells were extracted with 200 μl lysis buffer and luciferase activity was measured using a Monolight luminometer, model 3010 (Analytical Luminescence Laboratory, Sparks, MD). The results are expressed as relative AP-1 activity compared with untreated controls. Typical luminometer units for basal (untreated) AP-1 activity are designated 1.

ESR measurements

All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly as described in earlier studies (14,15). Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K3CrO8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. An EPR DAP 2.0 program was used for data acquisition and analyses. Reactants were mixed in test tubes in a total final volume of 450 μl. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations and measurements were made at room temperature and under ambient air except those specifically indicated otherwise. The receiver gain, time constant, sweep time, sweep width, modulation frequency, modulation amplitude and microwave power were set constant to allow relative intensity comparisons of spectra.

Oxygen consumption measurements

Because molecular oxygen is the potential source of $O_2^{-}\cdot$, the oxygen consumption was measured from vanadate-treated cells. Oxygen consumption was measured with an oxygraph equipped with a Clark microelectrode, model 516 (Gilson Medical Electronics, Middleton, WI). JB6 cells (1.0×10^6/ml) were pre-incubated at 37°C for 10 min and resting steady-state oxygen consumption was monitored over a period of 10 min. Vanadate- and NADPH-stimulated oxygen consumption was then determined in the absence or presence of vanadate for 10 min. The oxygraph was calibrated using medium equilibrated with gases of known oxygen content.

Statistics

Data were analyzed and are presented as means ± SD. Data were compared with controls using Student's *t*-test at a significance level of $P < 0.05$.

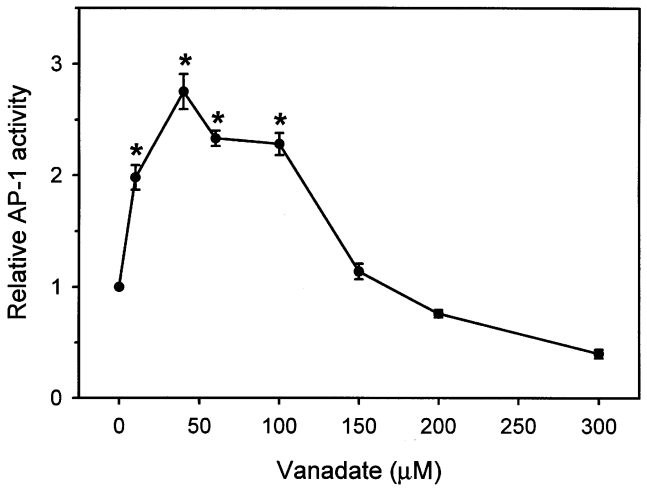


Fig. 1. Concentration dependence of vanadate-induced AP-1 activation in JB6 P^+ cells. AP-1 luciferase reporter plasmid-transfected cells JB6/AP/κB (5×10^4) suspended in 5% FBS MEM medium were seeded into each well of 24-well plates. After culturing at 37°C overnight, the cells were cultured in 0.5% FBS MEM medium for 24 h to minimize basal activity. Then the cells were exposed to the indicated concentrations of sodium vanadate. After culture for 24 h, the AP-1 activity was measured by luciferase activity assay as described in Materials and methods. Results are presented as relative AP-1 induction compared with untreated control cells as means ± SD of eight assay wells of two experiments. *Significant increase from control ($P < 0.05$).

Results

To test whether vanadate could induce AP-1 activation in cell culture, a promotion-sensitive (P^+) JB6/AP/κB cell line (26), which carries stably transfected nucleotides –73 to +63 of the collagenase promoter containing a single AP-1 site driving luciferase was used (25). Expression of the transgene has been shown to be stable for many passages (25). Vanadate dissolved in 1 ml EMEM medium was incubated with 5×10^4 cells for 24 h and the luciferase activity of the cell extract was tested. Figure 1 shows the dose-dependent induction of AP-1 activity by vanadate in JB6/AP/κB cells. Vanadate alone caused a significant dose-dependent AP-1 activation up to 40 μM concentration (Figure 1). A further increase in vanadate concentration above 40 μM resulted in inhibition of AP-1 activation. Vanadate-induced AP-1 activation also exhibited time dependence and the maximum activation of 2-fold occurred at 24 h incubation (Figure 2).

To further understand the mechanism of vanadate-induced AP-1 activation and the role of ROS, the effects of various reagents on vanadate-induced AP-1 activity were investigated. As shown in Figure 3, NADPH enhanced vanadate-induced AP-1 activation by >2-fold. Deferoxamine, which chelates the metal ions to make them less reactive, inhibited AP-1 activation by 47%. SOD, a scavenger of the $O_2^{-}\cdot$ radical, suppressed AP-1 activation by 44%. Catalase, which decom-

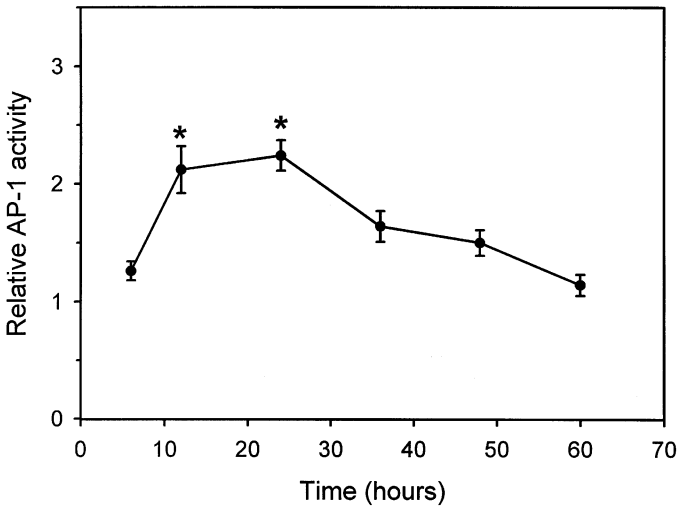


Fig. 2. Time course of vanadate-induced AP-1 activation in JB6/AP/κB cells. The incubation mixture contained 40 μM vanadate and JB6/AP/κB cells (5×10^4). Other experimental conditions were as described in the legend to Figure 1. Results are presented as relative AP-1 induction compared with untreated control cells. Values are means ± SD of eight assay wells of two experiments. *Significant increase from control ($P < 0.05$).

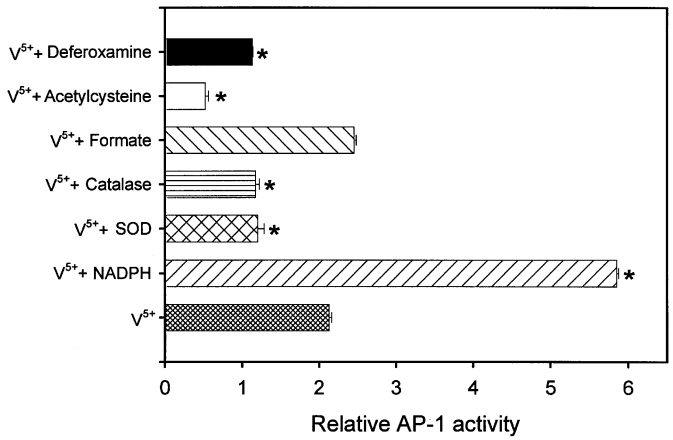


Fig. 3. Induction of AP-1 activity by vanadate in JB6/AP/κB cells and effect of various reagents. JB6/AP/κB cells (5×10^4) suspended in 5% FBS MEM medium were seeded into each well of 24-well plates. Then the cells were exposed to 40 μM sodium vanadate and various reagents as indicated. The concentrations of the reagents used were: NADPH, 200 μM; SOD, 500 U/ml; catalase, 500 U/ml; sodium formate, 50 mM; *N*-acetylcysteine, 5 mM; deferoxamine, 1 mM. Other experimental conditions were as described in the legend to Figure 1. Results are presented as relative AP-1 induction compared with untreated control cells. Each bar indicates the mean ± SD of three assay wells. The experiment was repeated three times. *Significant difference from vanadate alone ($P < 0.05$).

poses H_2O_2, decreased vanadate-induced AP-1 activation by 45%. *N*-acetylcysteine, an antioxidant, was most effective in blocking AP-1 activation induced by vanadate, inhibiting the response by 76%. Sodium formate, a ·OH radical scavenger, did not have significant effect.

In our earlier study (15), we showed that several cellular enzymes, such as glutathione reductase and lipoyl dehydrogenase, reduce vanadate to vanadium(IV) using NADPH as a cofactor. During the reduction process, molecular oxygen is reduced to $O_2^{-}\cdot$ and then to H_2O_2. Vanadium(IV) can react with H_2O_2 to produce ·OH. Figure 4 shows vanadate-induced AP-1 activation in the presence of NADPH and the effect of various antioxidants. As shown in Figure 3, vanadate-induced

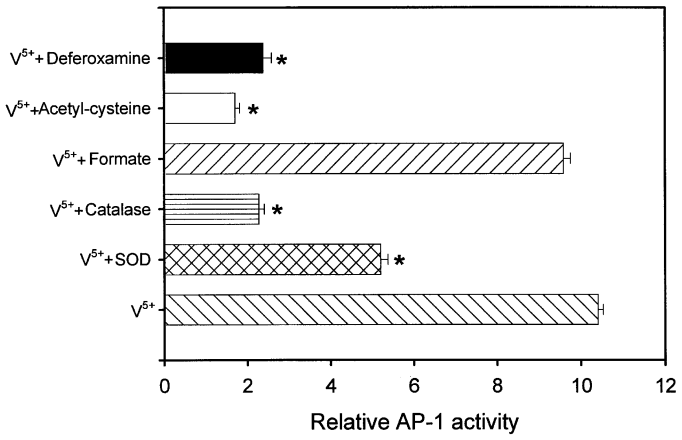


Fig. 4. Induction of AP-1 activity by vanadate in the presence of NADPH is inhibited by antioxidants. JB6/AP/κB cells (5×10^4) were treated with 40 μM sodium vanadate, 200 μM NADPH and various reagents as indicated. The concentrations of the reagents used were: SOD, 500 U/ml; catalase, 500 U/ml; sodium formate, 50 mM; *N*-acetylcysteine, 5 mM; deferoxamine, 1 mM. After culture for 24 h, the AP-1 activity was measured by luciferase activity assay as described in Materials and methods (20). Results are presented as relative AP-1 induction compared with untreated control cells. Each bar indicates the mean ± SD of three assay wells. The experiment was repeated three times. *Significant decrease from vanadate/NADPH alone ($P < 0.05$).

AP-1 activation is augmented in the presence of NADPH. However, this augmented AP-1 activation is still subject to modulation by various inhibitors. Both SOD and catalase decreased this augmented AP-1 activation, implicating $O_2^{-}\cdot$ and H_2O_2 in the activation process. Deferoxamine and *N*-acetylcysteine also inhibited vanadate-induced AP-1 activation in the presence of NADPH. Sodium formate, on the other hand, did not exhibit any significant inhibitory effect, thus excluding a role for ·OH in the activation of AP-1 by vanadate.

Previous studies have shown that members of the protein kinase C (PKC) family are critical for intracellular signaling pathways in various cell types, including TPA-induced transformation of JB6 cells (reviewed in ref. 36). To determine whether PKC is involved in AP-1 activation by vanadate, calphostin C, a specific inhibitor of PKC, was added to cell culture medium 1 h prior to addition of vanadate. Calphostin C has been reported to inhibit PKC by specifically interacting with its regulatory domain (37). As shown in Figure 5, calphostin C exhibited a dose-dependent inhibition of vanadate-induced AP-1 activation.

ESR spin trapping was used to examine free radical generation by cellular reduction of vanadate in the presence of NADPH. The use of spin trapping is necessary due to the reactive nature of the free radicals to be studied. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped and the hyperfine splittings of the spin adduct are generally characteristic of the original, short-lived, trapped radical. This method is specific and sensitive and is considered to be the preferred one for detection and identification of free radical generation. As shown in Figure 6a, a mixture of cells, vanadate and NADPH generated a relatively strong signal displaying a 1:2:2:1 quartet splitting indicative of a DMPO–OH adduct. SOD drastically decreased (~70–

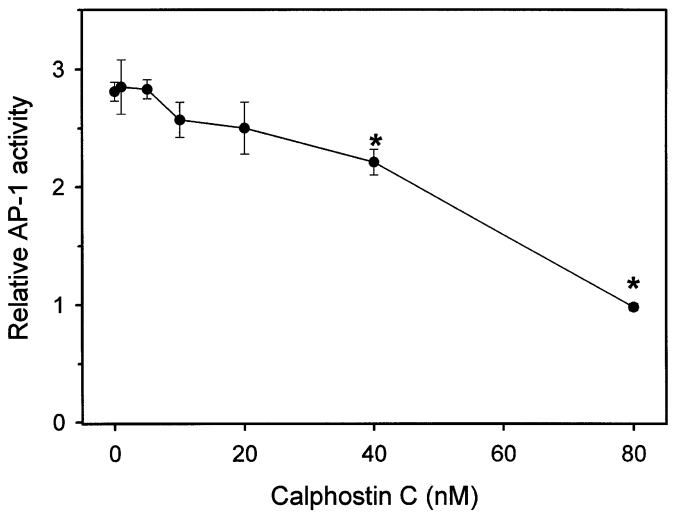


Fig. 5. PKC inhibitor calphostin C inhibits vanadate-induced AP-1 activation in JB6 P^+ cells. The incubation mixture contained JB6/AP/κB cells (5×10^4), 40 μM sodium vanadate and various concentrations of calphostin C. Calphostin C was added 1 h before treatment with vanadate. Other experimental conditions were as described in the legend to Figure 1. Results are presented as relative AP-1 induction compared with untreated control cells. Values are means ± SD of three assay wells. The experiment was repeated twice. *Significant decrease from vanadate alone ($P < 0.05$).

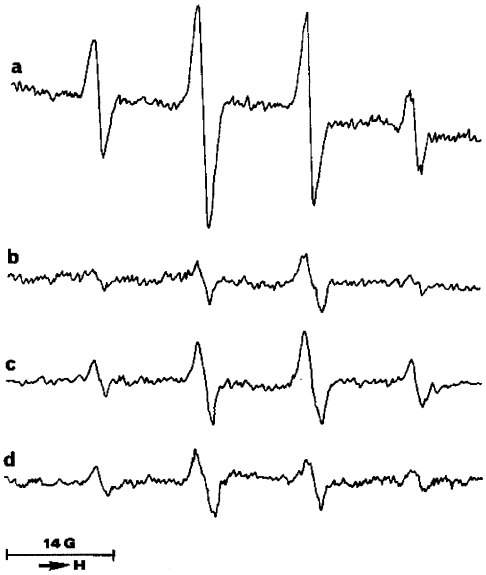


Fig. 6. ESR spectra obtained from incubation mixtures containing JB6/AP/κB cells (1.5×10^6), 100 mM DMPO and: (**a**) 1 mM sodium vanadate and 200 μM NADPH; (**b**) 1 mM sodium vanadate, 200 μM NADPH and 500 U/ml SOD (note the ~70–75% inhibition of spectral intensity); (**c**) 1 mM sodium vanadate, 200 μM NADPH and 500 U/ml catalase (note that catalase had a lesser effect); (**d**) 1 mM sodium vanadate. The spectra were recorded 3 min after incubation. The spectrometer settings were: receiver gain, 2.5×10^5; time constant, 0.3 s; modulation amplitude, 1.0 G; scan time, 8 min; magnetic field, 3340 ± 100 G.

75%) the signal intensity (Figure 6b), showing that the ESR signal observed in Figure 6a is dependent on $O_2^{-}\cdot$ generation. Catalase also decreased the signal intensity to a lesser level, suggesting that the observed signal was mostly attributable to $O_2^{-}\cdot$ (Figure 6c). Without NADPH, only a weak signal was observed (Figure 6d).

Figure 7 shows the generation of vanadium(IV) in the cellular reduction of vanadate in the presence of NADPH. Vanadium(IV) is paramagnetic and can be detected directly

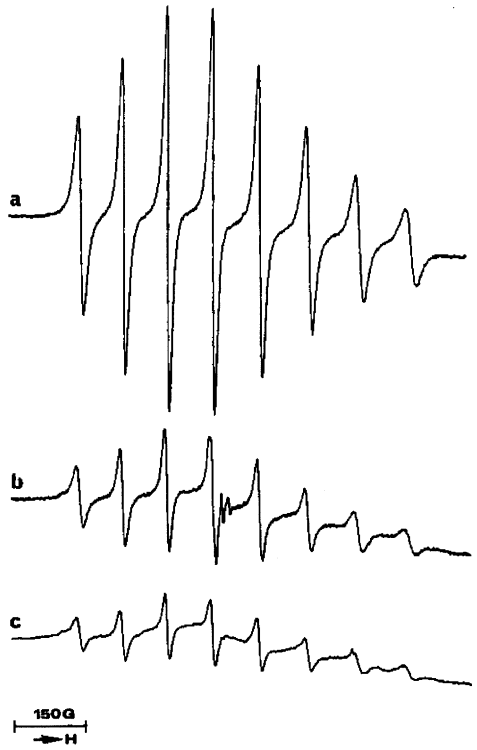


Fig. 7. (**a**) ESR spectrum recorded from incubation mixtures containing JB6/AP/κB cells (1.5×10^6), 1 mM sodium vanadate and 200 μM NADPH; (**b**) JB6/AP/κB cells (1.5×10^6) and 1.0 mM sodium vanadate; (**c**) 1.0 mM sodium vanadate and 200 μM NADPH. The spectra were recorded 3 min after incubation. The spectrometer settings were as described in the legend to Figure 6.

by ESR without using the spin trapping technique. Figure 7a shows the typical ESR spectrum obtained from a mixture of vanadate, cells and NADPH. This eight line spectrum with a hyperfine splitting (11 mT) is characteristic of vanadium(IV) (V^{51}, $I = 7/2$) (15). A mixture of vanadate and cells or a mixture of vanadate and NADPH generated a weak vanadium(IV) ESR signal (Figure 7b and c, respectively). Thus these results show that the reduction of vanadium(IV) is associated with disappearance of the signal.

The above results show that $O_2^{-}\cdot$ is generated in the cellular reduction of vanadate in the presence of NADPH. Exposure of JB6 P^+ cells to vanadate resulted in an increased rate of oxygen consumption above the control and addition of NADPH enhanced the oxygen consumption by JB6 P^+ cells, with a corresponding increase in vandium-induced $O_2^{-}\cdot$ and H_2O_2 production. Figure 8 shows the results of oxygen consumption measurements. Cells alone consumed molecular oxygen at a steady-state level of 1147 ± 104 nM/10^6 cells/h. Addition of vanadate and vanadate plus NADPH enhanced the oxygen consumption rate by 48 and 62%, respectively, thus implicating involvement of molecular oxygen as required in reduction.

Discussion

The results obtained in the present study show that vanadate is able to activate AP-1 and does so via PKC and $O_2^{-}\cdot$ and H_2O_2, but not via ·OH-mediated intracellular signaling reactions. The following results support these conclusion. (i) SOD inhibited vanadate-induced AP-1 activation. (ii) SOD inhibited the vanadate-dependent ESR spin adduct signal. Because SOD is specific for the $O_2^{-}\cdot$ radical, these inhibitory

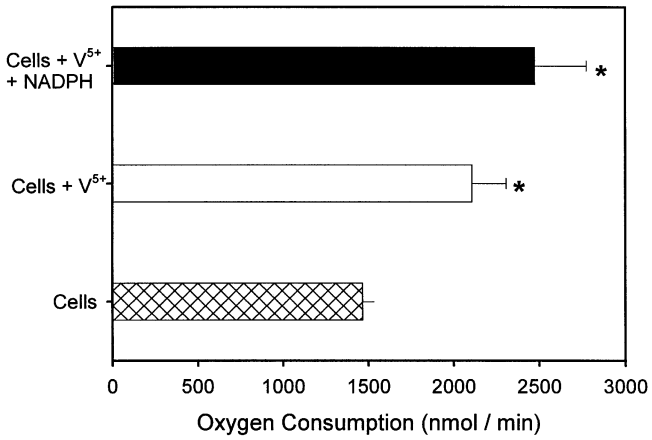
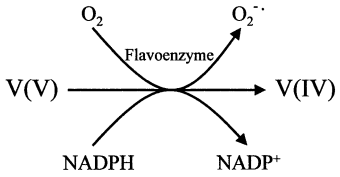


Fig. 8. Oxygen consumption of: (**a**) 1.0×10^6 JB6/AP/κB cells in pH 7.4 phosphate buffer solution; (**b**) 1.0×10^6 JB6/AP/κB cells + 40 μM sodium vanadate; (**c**) 1.0×10^6 JB6/AP/κB cells + 40 μM sodium vanadate + 200 μM NADPH. Each bar indicates the mean ± SD of three experiments. *Significant increase from cells alone ($P < 0.05$).

effects of SOD indicate a role of $O_2^{-}\cdot$ in vanadate-induced AP-1 activation. (iii) Catalase, an enzyme which decomposes H_2O_2, suppressed vanadate-induced AP-1 activation. (iv) Sodium formate, a specific ·OH radical scavenger, did not show a significant inhibitory effect. (v) NADPH increased vanadate-mediated $O_2^{-}\cdot$ and H_2O_2 generation and enhanced AP-1 activation. (f) both vanadate and NADPH enhanced the oxygen consumption rate of the cells. (g) Calphostin C, a specific inhibitor of PKC, inhibited activation of AP-1. In our earlier study (15), we showed that in the presence NAD(P)H several cellular flavoenzymes, glutathione reductase, lipoyl dehydrogenase and ferredoxin-$NADP^+$ oxidoreductase, are able to reduce vanadate to generate vanadium(IV). During the reduction process, molecular oxygen is consumed to generate $O_2^{-}\cdot$ and in the presence of SOD dismuted to H_2O_2. The reaction steps are described in the following reactions.



$$2O_2^{-}\cdot + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

These observations provide evidence to suggest that both $O_2^{-}\cdot$ and H_2O_2 are involved in vanadate-induced AP-1 activation. It may be noted that vanadium(IV) is able to generate ·OH radical from H_2O_2 via a Fenton-like reaction. Because formate did not inhibit AP-1 activation, this radical appears to be excluded as playing a significant role in vanadate-induced AP-1 activation.

AP-1 transactivation, which occurs during tumor promotion and progression in cell culture, appears to be a required event in neoplastic transformation promoted by phorbol esters and growth factors, but prior to the work reported herein it was unknown whether this might extend to transition metal tumor promoters (19–24,27,28). The AP-1 transcription factor consists of Jun/Jun homodimers or heterodimers of Jun (c-Jun, Jun B and Jun D) and Fos (c-Fos, Fos B, Fra-1 and Fra-2). AP-1 activity is regulated by a broad range of extracellular stimuli and functions as an intermediary transcriptional regulator in signal transduction processes leading to cell proliferation and transformation (19–23). Evidence suggesting the direct involvement of ROS in AP-1 activation has been obtained using defined ROS generating systems to challenge cultured cells. Oxidants, H_2O_2 and $O_2^{-}\cdot$, have been shown to induce c-*jun* and c-*fos* expression in a number of cell models (17,18,29–33,39–42). Although overexpression of c-Jun does not necessarily elevate AP-1 activity (34), when it does it causes cellular proliferation and/or transformation in those cells (19–24,43). TNFα and basic fibroblast growth factor are known to induce ROS production, which acts as a common signal to stimulate c-*fos* gene expression (44,45). Elucidating the possible causal role of AP-1 activation in transformation by vanadate will be of interest.

In intact cells, redox regulation of AP-1 activity occurs at the transcriptional and post-translational levels (45). The detailed mechanism of oxidant-induced AP-1 activation is still unclear. The involvement of an altered cellular thiol redox status was suggested for the induction of c-*fos* and c-*jun* expression caused by asbestos-induced oxidative stress (46). In support of this suggestion, high intracellular glutathione disulfide levels have been shown to be involved in AP-1 activation (47). The ROS generated by cellular reduction of vanadate may alter the thiol redox status and be responsible for vanadate-induced AP-1 activation. The inhibition of vanadate-induced AP-1 activation by the thiol-containing molecule *N*-acetylcysteine supports this suggestion.

It has been established that PKC modulates diverse cellular functions, including both proliferation and normal differentiation of many cell types (48). Selective oxidative transmembrane modulation of the regulatory and catalytic domains of PKC influences many cellular responses. While many questions remain to be answered concerning the thiol-containing molecule *N*-acetylcysteine supports this suggestion.

Temporal sequence of events, the results obtained from the present study show that calphostin C inhibited vanadate-induced AP-1 activation. This result suggests that PKC may be a critical, possibly ROS-independent component in cell signal pathways leading to proto-oncogene induction by vanadate.

The experimental evidence presented here provides support for a role of vanadate in ROS generation and their role as mediators in the activation of AP-1. Although these experimental studies are limited, they provide consistent changes commonly observed in human and rodent studies on carcinogenesis. Therefore, on the basis of these results, we derive the following conclusions: (i) vanadate is able to cause AP-1 activation in JB6 P^+ cells; (ii) this occurs via $O_2^{-}\cdot$ and H_2O_2-mediated reactions; (iii) the ·OH radical is not significantly involved in AP-1 activation and reduction of vanadate in intact JB6 P^+ cells to generate vanadium(IV); (iv) during the reduction process, molecular oxygen is reduced to $O_2^{-}\cdot$ and to H_2O_2 via dismutation; and (v) PKC is involved in the cell signal cascades leading to vanadate-induced AP-1 activation. Such evidence for vanadate-induced AP-1 activation via ROS-mediated reactions may improve our understanding of vanadate-modulated cell proliferation and differentiation, cell transformation, mutagenesis, proto-oncogene expression and phosphorylation of oncogene proteins.

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