

Vanadium-induced Nuclear Factor of Activated T Cells Activation through Hydrogen Peroxide*

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The present study investigated the role of reactive oxygen species (ROS) in activation of nuclear factor of activated T cells (NFAT), a pivotal transcription factor responsible for regulation of cytokines, by vanadium in mouse embryo fibroblast PW cells or mouse epidermal CI 41 cells. Exposure of cells to vanadium led to the transactivation of NFAT in a time- and dose-dependent manner. Scavenging of vanadium-induced H₂O₂ with N-acetyl-L-cysteine (a general antioxidant) or catalase (a specific H₂O₂ inhibitor) or the chelation of vanadate with deferoxamine, resulted in inhibition of NFAT activation. In contrast, an increase in H₂O₂ generation by the addition of superoxide dismutase or NADPH enhanced vanadium-induced NFAT activation. This vanadate-mediated H₂O₂ generation was verified by both electron spin resonance and fluorescence staining assay. These results demonstrate that H₂O₂ plays an important role in vanadium-induced NFAT transactivation in two different cell types. Furthermore, pretreatment of cells with nifedipine, a calcium channel blocker, inhibited vanadium-induced NFAT activation, whereas A23187 and ionomycin, two calcium ionophores, had synergistic effects with vanadium for NFAT induction. Incubation of cells with cyclosporin A (CsA), a pharmacological inhibitor of the phosphatase calcineurin, blocked vanadium-induced NFAT activation. All data show that vanadium induces NFAT activation not only through a calcium-dependent and CsA-sensitive pathway but also involved H₂O₂ generation, suggesting that H₂O₂ may be involved in activation of calcium-calcineurin pathways for NFAT activation caused by vanadium exposure.

Vanadium is a transition metal widely distributed in the environment. Occupational exposure to vanadium is common in oil-fired electrical generating plants and the petrochemical, steel, and mining industries (1). The available data from the literature provides evidence that in mammalian cell culture systems vanadium(V) is biologically more active than vanadium(IV) in inducing toxic effects (2). It has been reported that the cell can protect itself against vanadate toxicity by reduced glutathione-mediated conversion of vanadium(V) to vanadi-

um(IV), whereas vanadate(IV) does not undergo any change in valency state in BALB/3T3 cells (2). It has been found that vanadium-containing compounds exert potent toxic and carcinogenic effects, such as DNA damage and cell transformation (3–5). In animal studies, vanadium compounds or vanadium-containing air pollution particles have been shown to induce inflammation in the respiratory tract (6, 7). It has been reported that vanadium associated with air pollution particles, such as residual oil fly ash, can induce the synthesis and expression of inflammatory cytokines, such as IL-6,¹ IL-8, and TNF- α (6–9). Vanadium exposure *in vitro* was also found to cause the production of IL-1, TNF- α , and prostaglandin E2 (6, 10).

The nuclear factor of activated T cells (NFAT) was originally described as a transcriptional factor expressed in activated but not resting T cells (11–14). The induction of NFAT in T cells required a calcium-activated signaling pathway and was blocked by cyclosporin A (CsA) and FK506 (15–21). Over the last decade, studies from several laboratories have indicated that the pre-existing/cytoplasmic component of NFAT was a mixture of proteins belonging to a novel family of transcription factors (22–24). The first member of this family (NFATp, later renamed NFAT₁) was purified from cytoplasmic extracts of a murine T cell cloned by affinity chromatography using the distal NFAT site of murine IL-2 promoter (19, 25) and cloned from murine (Ar-5) and human (Jurkat) T cell cDNA libraries (25, 26). Other distinct proteins belonging to the same family, such as NFATc, NFAT₃, and NFAT₄, were also isolated and cloned (27–30). There are three functional domains in NFAT family proteins: the Rel-similarity domain, which is responsible for DNA-binding activity and interaction with AP-1; the NFAT-homology region, which regulates intracellular localization; and the transcriptional activation domain (31). The activation of NFAT in T cells includes dephosphorylation, nuclear translocation, and an increase in affinity for DNA binding (15). Stimuli that elicit calcium mobilization result in rapid dephosphorylation of NFAT proteins and their translocation to the nucleus. These dephosphorylated proteins show increased affinity for DNA binding (15).

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¹ The abbreviations used are: IL, interleukin; NFAT, nuclear factor of activated T cells; CsA, cyclosporin A; AP-1, activated protein-1; TPA, 12-O-tetradecanoylphorbol-13-acetate; NAC, N-acetyl-L-cysteine; NADPH, β -nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; DCFH-DA, dichlorofluorescein diacetate; HE, dihydroethidium; FBS, fetal bovine serum; MEM, Eagle's minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; ROS, reactive oxygen species; CMV, cytomegalovirus; PBS, phosphate-buffered saline; ESR, electron spin resonance; RLU, relative light unit(s); DMPO, 5,5-dimethyl-1-pyrroline N-oxide; GSK₃, glycogen synthase kinase-3; JNK, c-Jun N-terminal kinase.

Growing evidence indicates that NFAT is not only a T cell-specific transcriptional factor but also is expressed in a variety of lymphoid cells and in non-lymphoid tissue (15, 32, 33). NFAT involvement is reasonably well established for the production of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-18, TNF- α , interferon γ , and granulocyte macrophage-colony-stimulating factor in a variety of cell types (15). In addition, previous studies from our laboratory and other groups have shown that TNF- α , IL-6, and IL-8 are involved in vanadium-induced inflammation (6–10). Therefore, the objective of the present study was to determine if activation of NFAT occurred in response to vanadium and if so to investigate the molecular mechanism, by which lead to NFAT activation.

MATERIALS AND METHODS

Plasmids and Reagents—CMV-neo vector plasmid and NFAT-luciferase reporter plasmid were constructed as previously reported (14, 33–36). Sodium metavanadate (vanadate) and vanadyl sulfate trihydrate were purchased from Aldrich (Milwaukee, WI); deferoxamine, *N*-acetyl-L-cysteine (NAC), β -nicotinamide adenine dinucleotide phosphate (NADPH), superoxide dismutase (SOD), and sodium formate were purchased from Sigma Chemical Co. (St. Louis, MO); luciferase assay substrate was obtained from Promega (Madison); fetal bovine serum (FBS), Eagle's minimal essential medium (MEM), and Dulbecco's modified Eagle's medium (DMEM) were from BioWhittaker (Walkersville, MD). LipofectAMINE was from Life Technologies, Inc.; A23187, ionomycin, as well as cyclosporin A (CsA) were purchased from Alexis Biochemicals (San Diego, CA); dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (HE) were purchased from Molecular Probe (Eugene, OR).

Cell Culture—The JB6 P⁺ mouse epidermal cell line, Cl 41, and its transfectants, Cl 41 NFAT mass₁ and P⁺1-1, were established and cultured in monolayers at 37 °C, 5% CO₂ using MEM containing 5% FBS, 2 mM L-glutamine, and 25 μ g of gentamicin per ml as described previously (33, 37, 38). Mouse embryo fibroblast PW cells and its transfectants, PW NFAT mass₁, were cultured in DMEM with 10% FBS, 2 mM L-glutamine and 25 mg of gentamicin/ml.

Generation of Stable Co-transfectants—PW cells were cultured in a 6-well plate until they reached 85–90% confluence. 1 μ g of CMV-neo vector, with or without 12 μ g of NFAT-luciferase reporter plasmid DNA and 15 μ l of LipofectAMINE reagent, was used to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.033% trypsin and cell suspensions were plated onto 75-ml culture flasks and cultured for 24–28 days with G418 selection (800 μ g/ml). The stable transfectants were identified by measuring basal level of luciferase activity. Stable transfectant PW NFAT mass₁ were established and cultured in G418-free MEM for at least two passages before each experiment.

Assay for NFAT Activity in Vitro—Confluent monolayers of PW NFAT mass₁ or Cl 41 NFAT mass₁ were trypsinized, and 5×10^3 viable cells were suspended in 100 μ l of medium were added into each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were then exposed to vanadium for the indicated times and dosages. The cells were extracted with lysis buffer, and luciferase activity was measured as previously described (39). The results were expressed as NFAT activity relative to controls or relative luciferase unit (RLU) (33).

Cellular Superoxide (O_2^-) and H_2O_2 Staining Assay—Dihydroethidium (HE) is a specific O_2^- dye (40), while DCFH-DA has been frequently used to monitor H_2O_2 levels in cells (40). The cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with vanadate for 12 h. HE or DCFH-DA (both dissolved in Me₂SO and diluted with PBS to final concentrations of 5 μ M) was applied to the cells and incubated for another 15–20 min at 37 °C. The cells were washed twice with PBS and harvested for analysis by flow cytometry.

Electron Spin Resonance (ESR) Measurements—ESR spin trapping was used to detect short-lived free radical intermediates. 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 observed 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 trapped radical. ESR measurements were carried out using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured

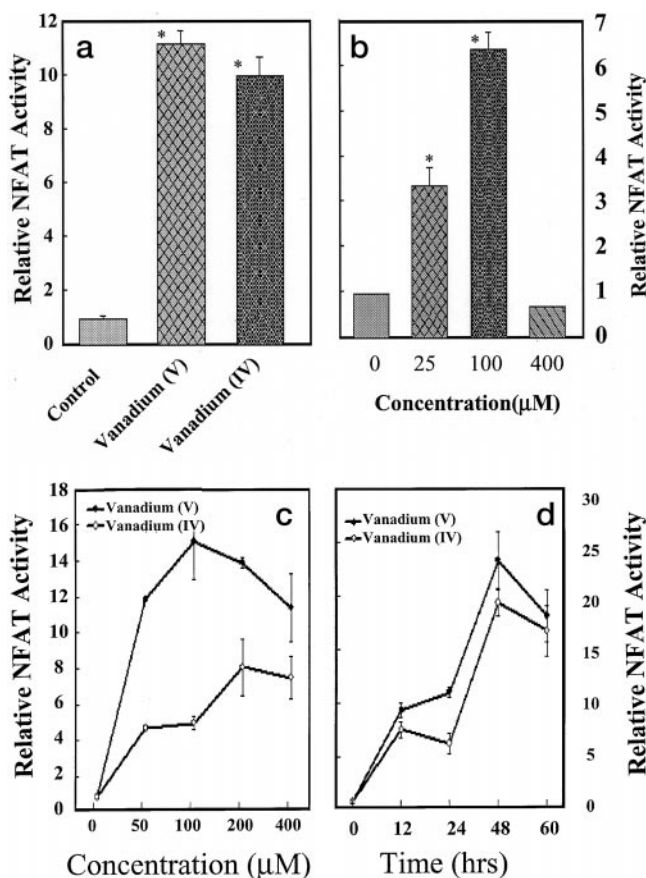


FIG. 1. Induction of NFAT-dependent transcription by vanadium in PW cells and Cl 41 cells. 8×10^3 cells of PW NFAT mass₁ (a, c, and d) or Cl 41 NFAT mass₁ (b) were seeded into each well of 96-well plates. After being cultured at 37 °C overnight, the cells were treated with: a, vanadium (100 μ M) for 24 h; b and c, for a dose-response study, the cells were exposed to different concentrations of vanadium as indicated for 24 h; d, for a time course study, the cells were exposed to 100 μ M vanadium for various times as indicated. Then, the luciferase activity was measured. Each bar indicates the mean and standard deviation of four repeat assay wells. The results are presented as NFAT-dependent transcription activity relative to control. The asterisk indicates a significant increase from control ($p < 0.05$).

(0.1 G) directly from magnetic field separation using potassium tetraper-oxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. PW cells (1×10^6) were mixed with 100 mM DMPO, 100 μ M NADPH, and 1 mM vanadate. The reaction mixture was then transferred to a flat cell for ESR measurement as described previously (2, 41).

RESULTS

Induction of NFAT Transactivation in PW Cells and Cl 41 Cells by Vanadium—To study the regulation of NFAT transcription activity by vanadium in cells, we generated a NFAT-luciferase reporter stable transfectant by co-transfecting the NFAT-luciferase reporter plasmid and CMV-neo plasmid into mouse fibroblast cells, PW NFAT mass₁. The results observed from this stable transfectant show that NFAT activities were markedly induced by exposure of PW cells to either vanadium(V) (increasing RLU from 3837 ± 183 to 42838 ± 1920) or vanadium(IV) (increasing RLU from 3837 ± 183 to 38321 ± 2839) ($p < 0.05$) (Fig. 1a). The activation of NFAT by vanadium appears to be time- and dose-dependent manner (Fig. 1, c and d). The maximum induction of NFAT activity by vanadium occurred between 36 and 48 h after cells were exposed to vanadium (Fig. 1d). To test whether vanadium-induced NFAT activation is cell-type-specific, we also exposed the Cl 41 NFAT mass₁ to vanadium. The results show that exposure of Cl 41 cells to vanadium resulted in significant activation of NFAT

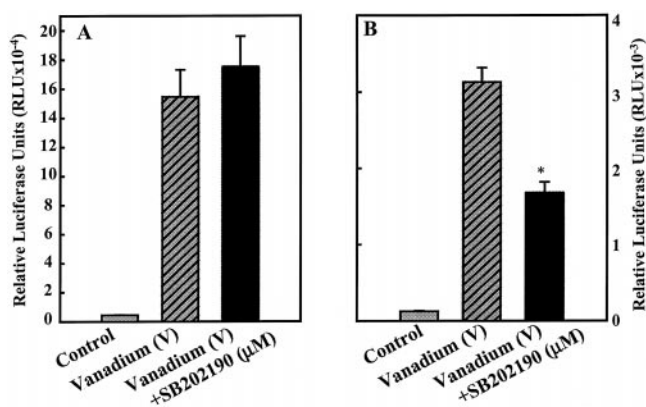


FIG. 2. Pretreatment of cells with SB202190 caused inhibition of vanadate-induced activation of AP-1, but NFAT. 8×10^3 cells of Cl 41 NFAT mass₁ (A) or P⁺1-1 (B) were seeded into each well of 96-well plates. After being cultured at 37 °C overnight, the cells were pretreated with 4 μ M SB202190 for 30 min. The cells were then treated with vanadate (100 μ M) for 24 h, and the luciferase activity was measured. Each bar indicates the mean and standard deviation of four repeat assay wells. The results are presented as relative luciferase unit (RLU). The asterisk indicates a significant decrease from vanadate control ($p < 0.05$).

activity at 25 and 100 μ M ($p < 0.05$) (Fig. 1b). These results demonstrate that vanadium is a stimulus for NFAT transactivation. This activation is not limited to a single cell type but appears to be a more generalized reaction to vanadium treatment.

It is well known that, in T cells, transactivation of NFAT is regulated tightly in response to elevations of both intracellular calcium ion (Ca^{2+}) and diacylglycerol following activation of phospholipase C (15). Increased intracellular calcium stimulates the activation of calmodulin (15). It is believed that binding of calmodulin to a region near the C terminus of calcineurin displaces the auto-inhibiting domain and exposes the calcineurin active site (15). Activated calcineurin subsequently dephosphorylates the cytoplasmic NFAT proteins, leading to NFAT nuclear translocation (16, 48). NFAT then forms a heteromeric transcriptional co-activator complex with AP-1 that co-induces NFAT-dependent transactivation (15). Because our previous data indicated that vanadium exposure also led to transactivation of AP-1 (3), the data shown above can not rule out the involvement of AP-1 in vanadium-induced NFAT transactivation even though the NFAT-luciferase reporter only contains the firefly luciferase under the control of three NFAT binding sites. To test this, we used a chemical inhibitor, SB202190, that can inhibit AP-1 activation by inhibition of AP-1 upstream kinase, p38 kinase. We find that pretreatment of cells with SB202190 does not show any inhibitory effects on vanadate-induced NFAT activation (Fig. 2A), whereas it markedly inhibits AP-1 activation induced by vanadate (Fig. 2B). These results suggest that vanadium-induced NFAT transactivation in PW cells is only dependent on NFAT, but not AP-1.

Reactive Oxygen Species Are Involved in NFAT Activation by Vanadium—Our previous studies have indicated that reactive oxygen species (ROS) are involved in vanadate-induced biological activities (42). If NFAT activation is responsible for some of the biological effects caused by vanadium, ROS generation may play a role in vanadium-induced NFAT activation. To test this possibility, ROS generation was determined in PW cells exposed to vanadium by both dye staining and ESR. The results show that PW cells alone did not generate any detectable amount of free radicals (Fig. 3A), whereas PW cells exposed to vanadate generated a strong ESR signal (Fig. 3B). The spectrum consists of a 1:2:2:1 quartet with hyperfine splittings of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and α -hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was

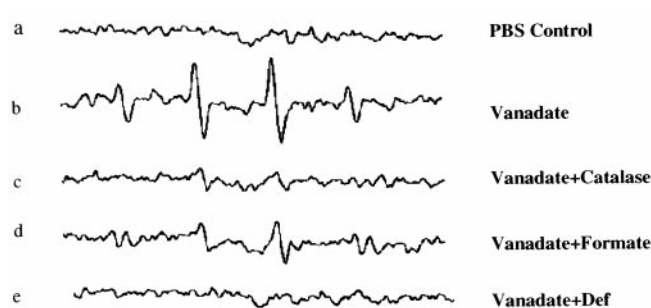


FIG. 3. Measurement of vanadate-induced ROS generation in PW cells by ESR. ESR spectra were recorded 6 min after mixing 1×10^6 PW cells, 100 mM DMPO, 1 mM sodium vanadate, and 100 μ M NADPH with or without different ROS scavengers as indicated. The final concentrations of these scavengers were: catalase, 2000 units/ml; or sodium formate, 100 mM. Deferoxamine, 2 mM, was used as a metal chelator.

assigned to the DMPO-OH adduct, which is evidence of $\cdot$ OH radical generation. Time course studies show that maximum generation of free radical is between 6.3 and 11.2 min after addition of vanadate (Fig. 4). Addition of catalase, a scavenger of H_2O_2 , inhibited $\cdot$ OH radical generation (Fig. 3C), indicating that H_2O_2 was produced in the vanadate-treated cells and involved in $\cdot$ OH generation. Addition of sodium formate, an $\cdot$ OH radical scavenger, decreased the signal intensity (Fig. 3D), further supporting the $\cdot$ OH radical generation. Incubation of the mixture with deferoxamine, a metal chelator, dramatically decreased the signal intensity (Fig. 3E). Measurements using HE, a specific fluorescent dye for O_2^- , or DCFH-DA, a fluorescent dye for H_2O_2 , demonstrate that incubation of cells with vanadium led to an increase in the generation of both O_2^- (increasing percentage of positive cells from 28.1% to 79.6%) and H_2O_2 (increasing percentage of positive cells from 34.6% to 87.5%) (Fig. 5). To investigate the possible role of ROS in NFAT activation by vanadium, the effects of specific modifiers of ROS on vanadium-induced NFAT activation were determined. The results show that pretreatment of cells with deferoxamine, NAC, or catalase caused inhibition of vanadium-induced NFAT activation ($p < 0.05$) (Fig. 6, a, b, and e), whereas addition of NADPH, which promotes ROS generation, or SOD, whose function is to scavenge O_2^- and generate H_2O_2 , enhanced vanadium-induced NFAT activation ($p < 0.05$) (Fig. 6, c and d). In contrast, treatment of cells with sodium formate did not inhibit vanadium-induced NFAT activation, suggesting that $\cdot$ OH may not play a significant role in vanadium-induced NFAT activation ($p > 0.05$) (Fig. 6f). These data support the hypothesis that H_2O_2 generation by vanadium is required for its activation of NFAT. We note that the time gap exists between the kinetics of ROS generation and NFAT induction. ROS generation reaches the peak between 6.3 and 11.2 min after exposure of cells to vanadate, whereas the maximum induction of NFAT activity is between 36 and 48 h post vanadate exposure. Because the NFAT activation is measured by using NFAT-luciferase reporter gene, so the time discrepancy not only includes the time for transmitting the signaling from extracellular stimulation to nuclear but also the luciferase gene transcription, translation as well as luciferase protein modifications. This explanation is supported by the observation that the maximum NFAT induction by H_2O_2 also needs 36–48 h after H_2O_2 exposure (data not shown).

Synergistic Effect of A23187 and Ionomycin on Vanadium-induced NFAT Activation—Ionomycin reportedly has a synergistic effect with TPA for NFAT transactivation in T and B cells, whereas ionomycin or TPA alone does not induce NFAT transactivation (14). Our previous studies have demonstrated that A23187 exhibited a synergistic effect with UV radiation

for NFAT induction (33). To test the effect of Ca^{2+} ionophore on vanadium-induced NFAT activation in PW cells, we incubated PW NFAT mass₁ with either A23187 or ionomycin. The results show that both A23187 and ionomycin exhibited a high synergistic augmentation of vanadium-induced NFAT activation ($p < 0.05$) (Fig. 7, *a* and *b*), whereas administration of A23187

or ionomycin alone did not show induction of NFAT activity in PW cells (Fig. 7, *a* and *b*). These synergistic effects appear to be dose-dependent (Fig. 7, *c* and *d*). These data suggest that elevation of intracellular calcium in PW cells plays a role in the activation of NFAT in response to vanadium.

Vanadium-induced NFAT Activation Is Dependent on Calcium Mobilization in PW Cells—To determine the role of calcium-dependent pathways in vanadium-induced NFAT activation, we investigated the effect of nifedipine, a calcium channel blocker, on vanadium-induced NFAT activation. Blocking calcium channel activity by pretreatment of PW cells with nifedipine resulted in inhibition of vanadium-induced NFAT activation ($p < 0.05$) (Fig. 8). These data suggest that activation of NFAT by vanadate and the synergistic effect of A23187 and ionomycin on vanadium-induced activation of NFAT both occur through calcium-dependent pathways.

Inhibition of Vanadium-induced NFAT Transactivation by Cyclosporin A (CsA)—Previous studies demonstrated that in T cells the major NFAT activation pathway appears to involve a calcium/calmodulin-dependent phosphatase, calcineurin (15, 16). To test the role of calcineurin in vanadate-induced NFAT-dependent transcription activity in PW cells and Cl 41 cells, cyclosporin A (CsA), a widely used pharmacological inhibitor of the phosphatase calcineurin, was used. Pretreatment of cells with CsA resulted in a dramatic inhibition of NFAT transactivation induced by vanadium ($p < 0.05$) in both PW (Fig. 9) and Cl 41 cells (date not shown). These data suggest that activation of calcineurin is required for vanadium-induced NFAT activation and that vanadium activates the NFAT transcription ac-

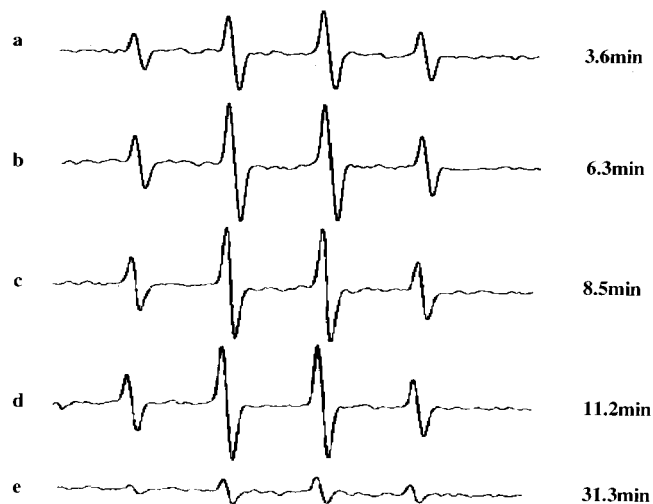


FIG. 4. Time course of vanadate-induced ROS generation in PW cells. ESR spectra were recorded at different time points after mixing 1×10^6 PW cells, 100 mM DMPO, 1 mM sodium vanadate, and 100 μ M NADPH.

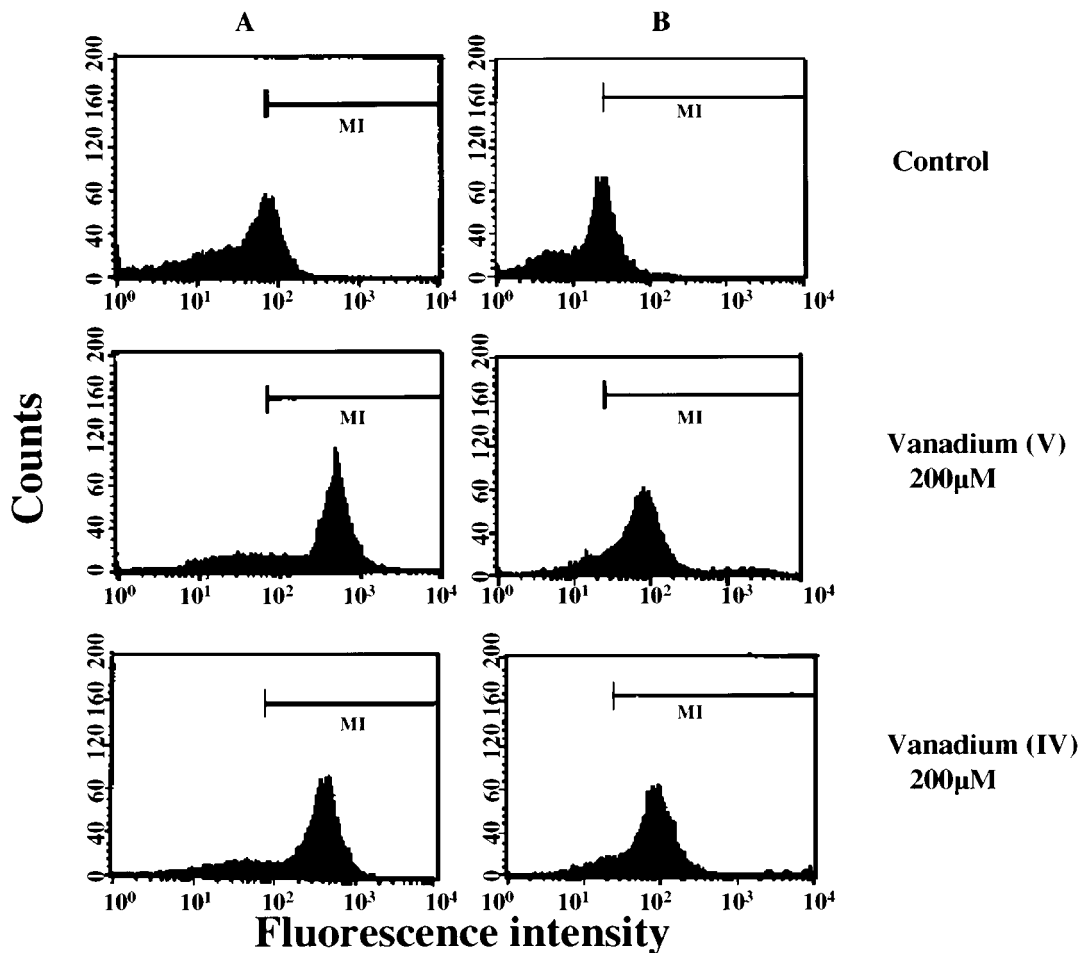


FIG. 5. Determination of O_2^- and H_2O_2 by HE and DCFH-DA staining in PW cells. PW cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with vanadium (200 μ M) for 60 min. HE (A) or DCFH-DA (B) was applied to the cells and incubated for another 15–20 min at 37 °C. The cells were washed twice with PBS and harvested for analysis by flow cytometry.

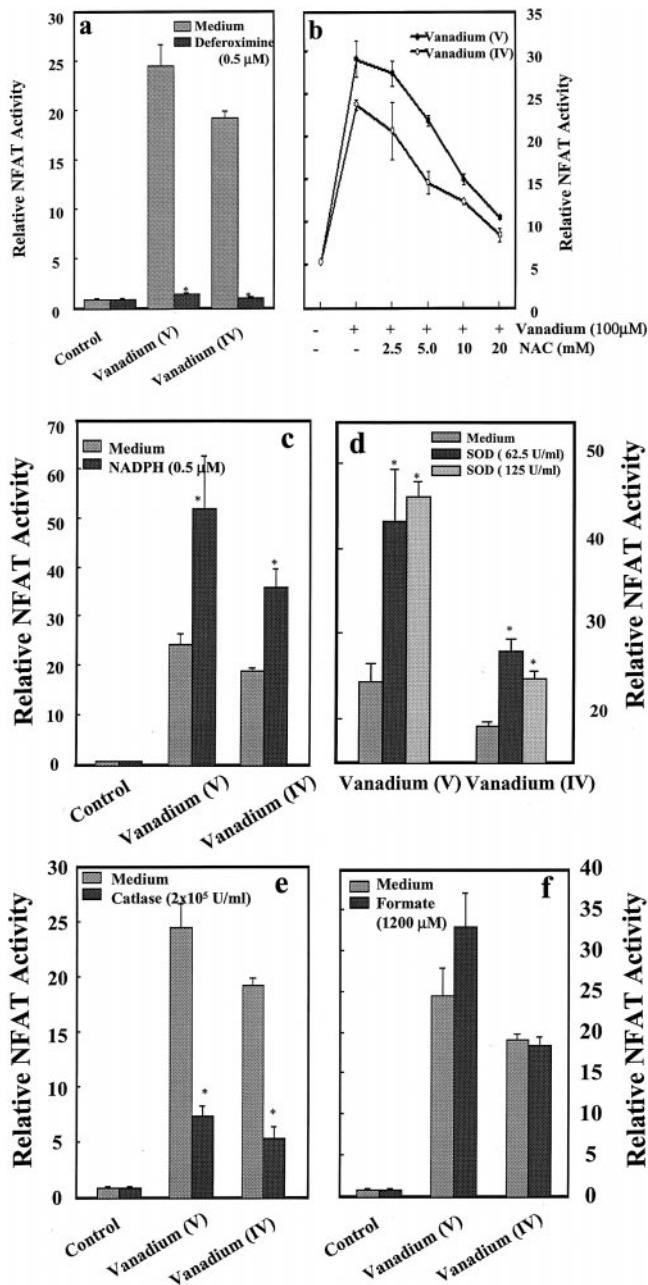


FIG. 6. Effects of free radical modifiers on NFAT activation by vanadium. PW NFAT mass₁ cells suspended in 10% FBS DMEM were added to each well of 96-well plates and cultured overnight. The cells were pretreated with different free radical modifiers at the concentrations indicated. The cells were then exposed to vanadium (100 μ M) for 24 h. The NFAT activity was determined by the luciferase activity assay. The results are presented as relative NFAT activity. Each column and bar indicates the mean and S.D. from triplicate assays. The asterisk indicates a significant increase (c and d) and a significant decrease (e) from vanadium alone ($p < 0.05$).

tivity in mouse embryo fibroblasts and epidermal cells through the pathway that is similar to that in T cells.

DISCUSSION

The antigen-regulated, cyclosporin A-sensitive nuclear factor of activated T cells (NFAT) is not only expressed in lymphoid cells as once thought but also expressed in other cells and organs, such as the skin and lung (15, 32). The stimuli and regulation of NFAT in cells other than lymphoid cells, however, remains unknown. In this report, we investigated the possible involvement of H_2O_2 in NFAT activation of fibroblasts and epidermal cells in response to vanadium. Exposure of cells to

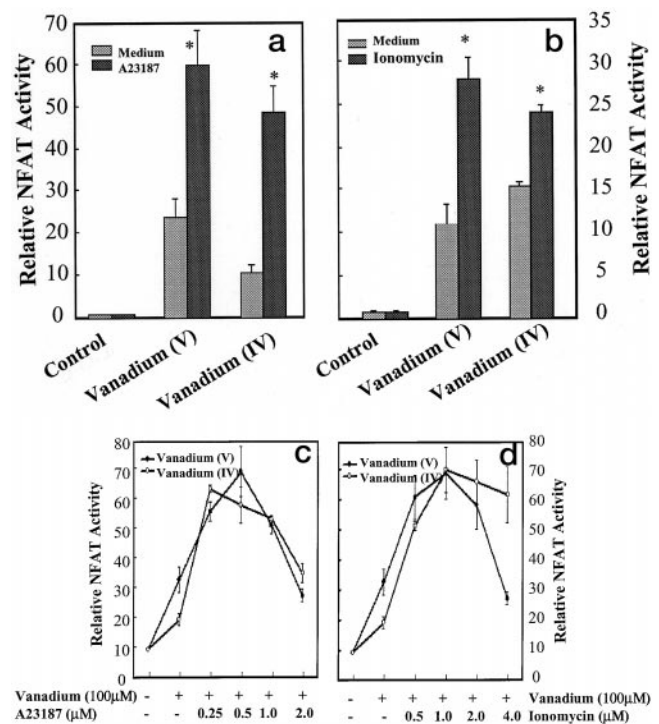


FIG. 7. Synergistic effect of A23187 and ionomycin on vanadium-induced NFAT activity. 8×10^5 PW NFAT mass₁ cells suspended in 10% FBS DMEM medium were added to each well of 96-well plates. After being cultured at 37 $^{\circ}$ C overnight, the cells were treated with (a) vanadium (100 μ M), A23187 (1 μ M), or vanadium (100 μ M) + A23187 (1 μ M), or (b) vanadium (100 μ M), ionomycin (1 μ M), or vanadium (100 μ M) + ionomycin (1 μ M). For the dose-response study, the cells were exposed to vanadium (100 μ M) plus different concentrations of A23187 (c) or ionomycin (d). The cells were harvested after being cultured for 48 h. The NFAT activity was measured by luciferase activity assay. The results are presented as relative NFAT-dependent transcription activity. Each bar indicates the mean and standard deviation of assays from triplicate wells. The asterisk indicates a significant increase from vanadium alone ($p < 0.05$).

vanadium caused marked increases in NFAT-dependent transcription in a time- and dose-dependent manner. Co-stimulation studies show that A23187 or ionomycin augments the NFAT-mediated transcription synergistically in response to vanadium. Pretreatment of cells with nifedipine or CsA results in impairment of NFAT transactivation induced by vanadium. These results strongly suggest that vanadium is able to induce NFAT activation. Furthermore, scavenging of vanadium-induced H_2O_2 by NAC or catalase, or the chelation of vanadate by deferoxamine resulted in inhibition of NFAT activation, whereas an increase in H_2O_2 generation in response to SOD or NADPH enhanced vanadium-induced NFAT activation. These results suggest that vanadium induces NFAT transactivation is dependent on H_2O_2 generation caused by vanadium.

NFAT isoforms are expressed in different tissues. It has been reported that NFAT₁ and NFAT₂ mRNAs have been detected in brain, heart, skeletal muscle, testis, placenta, pancreas, small intestine, prostate, colon, skin tumors, as well as in lung (15, 32). NFAT expression or NFAT-derived transactivation has also been described in several types of nonlymphoid cells, including mast (43), endothelial (44), neuronal (45), vascular smooth muscle (46), and liver-derived Chang cells (47). In this study, we have found that exposure of either mouse embryo fibroblasts (PW cells) or mouse epidermal cells (Cl 41) resulted in marked activation of NFAT-dependent transcription. These results are not only consistent with previous findings that NFAT mRNA is detectable in skin (32) but also for the first time provide the evidence that NFAT is expressed in embryo

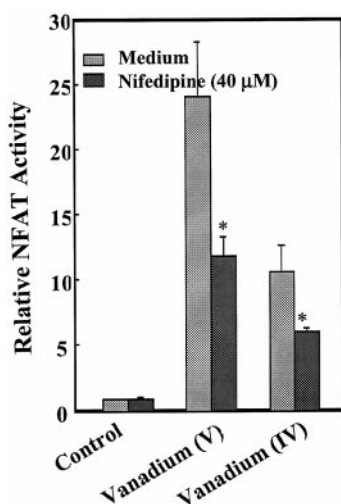


FIG. 8. Inhibition of vanadium-induced NFAT activity by pretreatment of cells with nifedipine. 8×10^3 PW NFAT mass₁ cells suspended in 10% FBS DMEM were added to each well of 96-well plates. After being cultured at 37 °C overnight, the cells were first treated with 40 μM nifedipine for 30 min and sequentially were exposed to vanadium (100 μM). After being cultured for 48 h, the cells were harvested, and the NFAT activity was measured by luciferase activity assay. The results are presented as relative NFAT-dependent transcription activity. Each bar indicates the mean and standard deviation of assays from triplicate wells. The asterisk indicates a significant decrease from vanadium alone ($p < 0.05$).

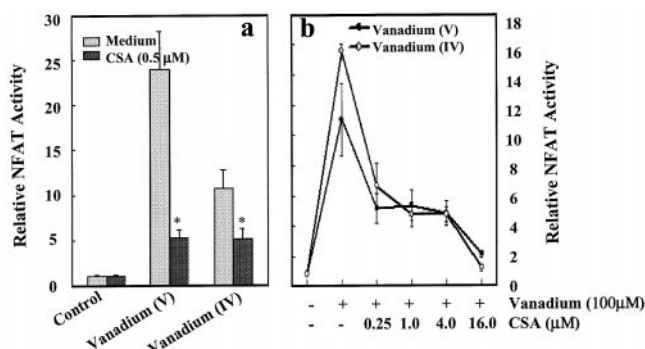


FIG. 9. Blocking vanadium-induced NFAT transactivation by cyclosporin A. PW NFAT mass₁ were seeded to each well of 96-well plates and cultured until 90% confluent. The cells were then treated with cyclosporin A at (a) 0.5 μM or (b) at the concentration as indicated for 30 min and sequentially were exposed to 100 μM vanadium. After being cultured for 48 h, the cells were harvested and the NFAT activity was measured by luciferase activity assay. Each bar indicates the mean and standard deviation of assays from triplicate wells. The asterisk indicates a significant decrease from vanadium alone ($p < 0.05$).

fibroblasts, suggesting that NFAT may play some role in embryonic development. The activation of NFAT includes dephosphorylation, nuclear translocation, and an increase in affinity for DNA binding (15). Stimuli that elicit calcium mobilization result in rapid dephosphorylation of NFAT proteins and their translocation to the nucleus. NFAT then binds to the promoter region of its target genes and initiates the transcription of these target genes, in turn leading to translation (15). Therefore, the translocation of NFAT occurs within 3 h (33), whereas its target gene proteins could be detected between 24 and 48 h after cells exposed to stimulus (15). The system used in this study is NFAT-luciferase reporter cDNA, which includes the firefly luciferase cDNA under the control of NFAT regulatory element (promoter region of mouse IL-2, a typical target gene of NFAT). Thus, the NFAT activation by vanadium appears at 12 h and reaches peak around 36–48 h after cells exposed to vanadium.

In T cells, transactivation of NFAT is regulated tightly in

response to elevations of both intracellular calcium ion (Ca^{2+}) and diacylglycerol following activation of phospholipase C (15). Increased intracellular calcium stimulates the activation of calmodulin (15). It is believed that binding of calmodulin to a region near the C terminus of calcineurin displaces the auto-inhibiting domain and exposes the calcineurin active site (15). Activated calcineurin subsequently dephosphorylates the cytoplasmic NFAT proteins, leading to NFAT nuclear translocation (15, 16, 48). NFAT forms a heteromeric transcriptional co-activator complex with AP-1 that co-induces NFAT-dependent transactivation (15). It has also been reported that phosphorylation of NFAT is regulated by several protein kinases, including GSK₃ and JNK₂ (15, 49–51). Other than lymphocytes, NFAT activation has been demonstrated in only a few cell types, such as vascular smooth muscle cells following induction with platelet-derived growth factor and liver-derived Chang cells following exposure to hepatitis B virus × protein (46, 47). The results obtained from the present investigation show that vanadium alone could induce an increase of NFAT activity of more than 10-fold. It appears that vanadium alone is the strongest NFAT inducer reported thus far. Recently, we reported (33) that UV irradiation resulted in activation of NFAT-mediated transcription through a calcium-dependent pathway in mouse epidermal JB6 cells as well as in mouse skin. The present study found that A23187 or ionomycin augmented the NFAT-mediated transcription synergistically in combination with vanadium. Pretreatment of cells with nifedipine or CsA resulted in impairment of NFAT transactivation induced by vanadium. Therefore, the vanadium-induced NFAT transactivation appears to require an increase in free intracellular calcium and calcineurin activation.

Vanadium-containing compounds exert potent toxic and carcinogenic effects, such as cell transformation (3–5). It has been demonstrated that vanadium-mediated generation of reactive oxygen species (ROS) is responsible for most biological effects caused by vanadium (41, 42). It is well accepted that extracellular stimuli trigger signals through a cascade of protein-protein interactions (52–54). It is generally believed that these extracellular stimuli generate and/or require reactive free radicals or derived oxidant species to successfully transmit their signals to the nucleus (53, 55). In addition to inducing cellular injury, such as DNA damage and lipid peroxidation, ROS also function as intracellular messengers (2, 53, 56). Accumulating data suggest a vital role of ROS in mediating cellular responses by various extracellular stimuli (2, 40–42, 53, 56). It has been reported that generation of H_2O_2 is required for platelet-derived growth factor signal transduction (57). Involvement of ROS in NFAT activation in T lymphocytes was first demonstrated by the observation that treatment of T cells with dithiocarbamate resulted in inhibition of NFAT activation and NFAT-mediated cytokine gene expression (58, 59). The results presented here demonstrate that increased intracellular H_2O_2 levels are associated with induction of NFAT activity by vanadium. The following experimental observations support this conclusion: (a) the ESR spectrum and dye staining show that vanadium induces ROS generation in cells; (b) catalase, a scavenger of H_2O_2 , inhibited NFAT activation induced by vanadium; (c) sodium formate, an $\cdot OH$ radical scavenger, did not affect vanadium-induced NFAT activation; (d) SOD, which scavenges O_2^- and generates H_2O_2 , enhanced NFAT activation by vanadium; (e) NADPH, which promotes H_2O_2 generation, also enhanced NFAT activation by vanadium; (f) deferoxamine, a metal chelator, dramatically decreased the NFAT activation induced by vanadate, indicating a key role for vanadate in ROS generation and NFAT activation. Thus, H_2O_2 is involved in vanadium-induced NFAT activation. Considering the results that calcium-calcineurin pathway is also required for vanadi-

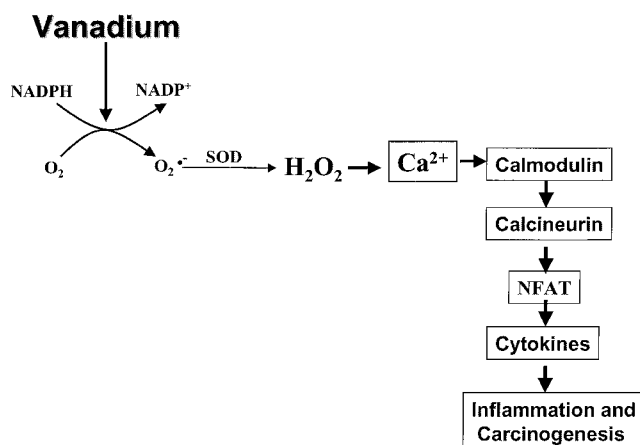


FIG. 10. The model of signal transduction pathway for NFAT activation induced by vanadium.

um-induced NFAT activation, we speculate that H_2O_2 may be an initiator for calcium-calcineurin pathway for NFAT activation (Fig. 10). This hypothesis was strongly supported by the previous findings that exposure of cells to H_2O_2 induces early release of Ca^{2+} from mitochondria into cytosol, which was mediated by annexin VI translocation and inactivation of plasma membrane Ca^{2+} -ATPase (60).

NFAT is a transcription factor, which has been reported to play an essential role in IL-2 gene expression (14). Binding sites for NFAT have also been found within the promoter regions of cytokines, including granulocyte macrophage-colony-stimulating factor, TNF- α , IL-1, IL-3, IL-4, IL-5, and IL-8 (15, 47, 50). Previous studies have indicated that expression of IL-8, TNF- α , and other cytokines is associated with the initiation and control of effective immune and inflammatory responses and may be related to cancer development (47, 50). Therefore, we suggest that NFAT is involved in vanadium-induced inflammation and may subsequently be involved in the carcinogenic effects of vanadium. This idea is supported by previous findings that CsA and FK506, the two most commonly used inhibitors for NFAT activation, exhibit strong anti-tumor promotion activity by specifically only targeting and blocking the activation of a Ca^{2+} -dependent phosphatase, calcineurin (61, 62).

In conclusion, we demonstrate that NFAT is expressed in embryonic cells and vanadium is a strong inducer of NFAT activation. Vanadium induces NFAT activation through CsA-sensitive and calcium-dependent signal transduction pathways, which required H_2O_2 generation caused by vanadium. Transactivation of NFAT in cells other than lymphocytes supports the hypothesis that NFAT activation may play an important role in vanadium-induced biological effects, such as inflammation and tumor promotion, possibly through NFAT-mediated expression of inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8, and TNF- α .

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Vanadium-induced Nuclear Factor of Activated T Cells Activation through Hydrogen Peroxide

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