

Vanadate Induces p53 Transactivation through Hydrogen Peroxide and Causes Apoptosis*

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Vanadium is a metal widely distributed in the environment. Although vanadate-containing compounds exert potent toxic effects on a wide variety of biological systems, the mechanisms controlling vanadate-induced adverse effects remain to be elucidated. The present study investigated the vanadate-induced p53 activation and involvement of reactive oxygen species (ROS) in p53 activation as well as the role of p53 in apoptosis induction by vanadate. Exposure of mouse epidermal JB6 cells to vanadate led to transactivation of p53 activity in a time- and dose-dependent manner. It also caused mitochondrial damage, apoptosis, and generated ROS. Scavenging of vanadate-induced H₂O₂ by N-acetyl-L-cysteine (a general antioxidant) or catalase (a specific H₂O₂ inhibitor), or the chelation of vanadate by deferoxamine, resulted in inhibition of p53 activation and cell mitochondrial damage. In contrast, an increase in H₂O₂ generation in response to superoxide dismutase or NADPH enhanced these effects caused by vanadate. Furthermore, vanadate-induced apoptosis occurred in cells expressing wild-type p53 (p53+/+) but was very weak in p53-deficient (p53-/-) cells. These results demonstrate that vanadate induces p53 activation mainly through H₂O₂ generation, and this activation is required for vanadate-induced apoptosis.

The p53 tumor suppressor protein is a transcription factor that enhances the transcriptional rate of several genes known to play a critical role in transducing signals from DNA damage (1–5). It is elevated in response to genotoxic agents, such as ionizing radiation, UV light, or certain chemicals (1, 4, 6). The activation of p53 has been implicated in cell cycle control, DNA repair, and apoptosis (5–7). The function of p53 is regulated at the levels of transcription, translation, protein turnover, and cellular compartmentalization, as well as association with other proteins (8). In addition, growing evidence indicates that the ability of p53 to inhibit diverse regulatory functions is likely to depend on its phosphorylation, which is conformation-dependent (8, 9). p53 phosphorylation is mediated by a variety of protein kinases, including casein kinase I, casein kinase II, protein kinase A, CDK7, DNA-activated protein kinase, protein

kinase C, c-Jun NH₂-terminal kinases, extracellular signal-regulated kinases, and p38 kinase (8–10).

Apoptosis, or programmed cell death, has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions (1–4, 11, 12). It is essential in many physiological processes, including maturation and effector mechanisms of the immune system, embryonic development, and hormone-dependent tissue remodeling (11–15). Inappropriate regulation of apoptosis may play an important role in many pathological conditions such as hepatotoxicity, ischemia, stroke, heart disease, cancer, AIDS, autoimmunity, and degenerative diseases of the central nervous system (16–18).

Vanadium is a transition metal widely distributed in environment. Occupational exposure to vanadium is common in oil-fired electrical generating plants and the petrochemical, steel, and mining industries (19, 20). It has been found that vanadate-containing compounds exert potent toxic and carcinogenic effects, such as DNA damage and cell transformation (21–23). Normally, if the cell is damaged by external agents, such as vanadate, it will respond to such damage by activating signal transduction pathways that control the activation of transcription factors and the regulation of gene expression as well as transiently delaying cell cycle progression to allow the repair of damaged DNA. If the cell damage is severe and cannot be repaired, the cells will undergo apoptosis. Therefore, apoptosis plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged or improperly proliferating cells. Investigation of the mechanism of carcinogen-induced apoptosis is very important for understanding overall carcinogenesis. It has been demonstrated that vanadate-mediated generation of reactive oxygen species (ROS)¹ plays an important role in its adverse biological effects (24–27). Our previous studies also indicate that generation of H₂O₂ by vanadate is a mediator for apoptosis induction in a cell culture model (24). The present study investigated the p53 transactivation and its mechanisms as well as its role in apoptosis induction by vanadate.

MATERIALS AND METHODS

Reagents—Sodium metavanadate (vanadate) was purchased from Aldrich; deferoxamine, N-acetyl-L-cysteine (NAC), NADPH, superoxide dismutase (SOD), and sodium formate were purchased from Sigma;

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¹ The abbreviations used are: ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; SOD, superoxide dismutase; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; DCFH-DA, 2',7'-dichlorofluorescein diacetate; HE, dihydroethidium; FBS, fetal bovine serum; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline.

JC-1, DiOC₆, DCFH-DA, and dihydroethidium (HE) were purchased from Molecular Probes (Eugene, OR); luciferase assay substrate was obtained from Promega; fetal bovine serum (FBS), Eagle's minimal essential medium (MEM), and Dulbecco's modified Eagle's medium, as well as RPMI 1640 were from BioWhittaker.

Cell Culture—The JB6 P⁺ mouse epidermal cell line (Cl 41) and its stable p53 luciferase reporter plasmid transfectant (Cl 41 p53 cells) were cultured in monolayers at 37 °C under 5% CO₂ using MEM containing 5% fetal calf serum, 2 mM L-glutamine, and 25 µg/ml gentamicin (28–30). Normal embryo fibroblasts (p53^{+/+}) or p53-deficient embryo fibroblasts (p53^{-/-}) were cultured in Dulbecco's modified Eagle's medium with 10% FBS, 2 mM L-glutamine, and 25 µg/ml gentamicin (31).

Assay for p53 Activity—Confluent monolayers of Cl 41 p53 cells were trypsinized, and 8 × 10³ viable cells were suspended in 100 µl 5% FBS/MEM. The cells were added into each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Twelve to 24 h later, cells were starved by culturing them in 0.1% FBS/MEM for 12 h. The cells were exposed for 24 h to different concentrations of vanadate for p53 induction. The cells were extracted with lysis buffer, and luciferase activity was measured using a luminometer (Monolith 3010). The results were expressed as p53 activity relative to controls (5, 6, 30).

DNA Fragmentation Assay—Cl 41 cells were exposed to ultraviolet C (60 J/m²) or treated with different concentrations of vanadate for 24 h. All the cells were harvested by centrifugation and lysed with a lysis buffer (5 mM Tris-HCl, pH 8.0; 20 mM EDTA; 0.5% Triton X-100) on ice for 45 min. Fragmented DNA in the supernatant (after a centrifugation at 14,000 rpm for 30 min at 4 °C) was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and once with chloroform before precipitating with ethanol and salt. The DNA pellet was washed once with 70% ethanol and resuspended in TE buffer, pH 8.0, with 100 µg/ml RNase at 37 °C for 2 h. The DNA fragments were separated by 1.8% agarose gel electrophoresis and visualized under UV light as described previously (5, 6).

DNA Fragment End Labeling Assay—The cells were treated with vanadate for 24 h. The cells were then harvested. The DNA fragment end labeling assays were performed as described in the protocol by the manufacturer using an *in situ* cell death detection kit employing fluorescein (Roche Molecular Biochemicals). The cells were analyzed by flow cytometry.

Mitochondria Transmembrane Potential ($\Delta\psi_m$) Assay—JC-1 and DiOC₆ are two specific fluorescent dyes used to test the cell mitochondrial membrane potential (32). The cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with vanadate for 12 h. The dye, JC-1 or DiOC₆ (dissolved in Me₂SO and diluted with PBS to final concentrations of 10 µg/ml or 40 nM, respectively), 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.

Cellular Superoxide (O₂⁻) and H₂O₂ Staining Assay—HE is a specific O₂⁻ dye (32), and DCFH-DA has been frequently used to monitor H₂O₂ levels in cells (32). 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 and 5 µM, respectively) 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 measurements were carried out using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K₃CrO₈) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. Cl41 cells (1 × 10⁶) were mixed with 100 mM 5,5-dimethyl-1-pyrroline N-oxide, 100 µM NADPH, and 1 mM vanadate. The reaction mixture was then transferred to a flat cell for ESR measurement as described previously (19, 26).

RESULTS

Activation of p53 Transactivation Activity by Vanadate—To investigate the possible activation of p53 by vanadate, we exposed the well characterized JB6 cell with PG13-luciferase reporter stable transfectants to vanadate (5, 6, 30). The results show that vanadate markedly activated p53-dependent transcription activity in a time- and dose-dependent manner (Fig. 1). The maximum induction of p53 activity occurred between 36 and 48 h after cell exposed to vanadate (Fig. 1B). These results

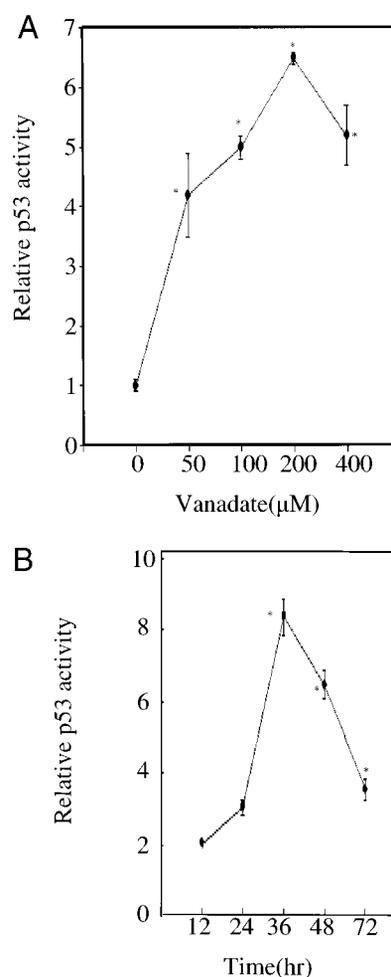


FIG. 1. Induction of p53-dependent transcription activity by vanadate. JB6, Cl 41, and PG13-luciferase (PG13-luc) stable transfectant (Cl 41 p53) cells, suspended in 5% FBS/MEM, were added to each well of 96-well plates and cultured overnight. **A**, for the dose-response study, the cells were treated for 30 h with vanadate at the concentrations indicated. **B**, for time course study, the cells were treated with 200 µM vanadate for various time points as indicated. The asterisk indicates a significant increase from control ($p < 0.05$). p53 activity was determined by the luciferase activity assay. The results are presented as relative p53 activity. Each point and bar indicates the mean \pm S.D. from triplicate assays.

demonstrated that vanadate is a stimulus for p53 transactivation. It may be noted that these data are different from previous reports that indicate that at a concentration of 1 mM, vanadate decreased (35), whereas at a concentration of 10 µM, vanadate did not exhibit any observable (36) effects on p53 mRNA levels in human cancer HeLa cells and C127 mouse tumor cells, respectively. These differences may be due to different cell lines and doses used among these studies. This was supported by our study that 10 µM vanadate had no effects on p53 activity in JB6 cells (data not shown).

Generation of Reactive Oxygen Species Is Required for p53 Activation by Vanadate—It has been reported that vanadate may generate ROS under some circumstances (24). To study the relationship between ROS generation and p53 activation, vanadate-induced ROS production was determined either by dye staining or ESR. Fig. 2A showed that cells alone did not generate any detectable amount of free radicals, whereas a mixture of cells and vanadate generated a strong ESR spectrum (Fig. 2B). 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 -hy-

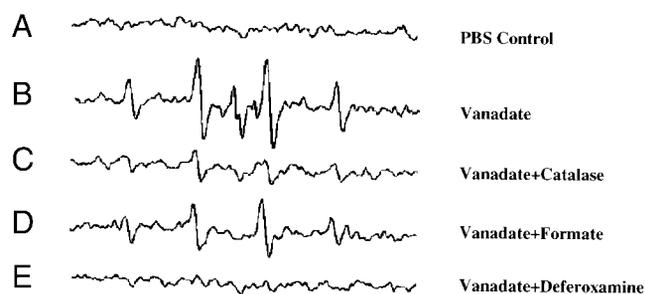


FIG. 2. Measurement of vanadate-induced ROS generation by ESR. ESR spectra were recorded 6 min after mixing 1×10^6 Cl 41 cells, 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide, 1 mM sodium vanadate, and 100 μ M NADPH with or without different ROS scavengers as indicated. The final concentrations of these scavengers were catalase, 2000 units/ml; SOD, 0.5 μ g/ml; or sodium formate, 100 mM. Deferoxamine, 2 mM, was used as a metal chelator.

drogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO-OH adduct, which is evidence of \cdot OH radical generation. Addition of catalase, a scavenger of H_2O_2 , inhibited \cdot OH radical generation (Fig. 2C), indicating that H_2O_2 was produced in the vanadate-treated cells and served as a precursor of \cdot OH generation. Addition of sodium formate, an \cdot OH radical scavenger, decreased the signal intensity (Fig. 2D), confirming that the 1:2:2:1 quartet observed in this study was due to \cdot OH generation. Incubation of the mixture with deferoxamine, a metal chelator, dramatically decreased the signal intensity (Fig. 2E), indicating a key role of vanadate in the radical generation. 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 vanadate led to an increase in the generation of both O_2^- (increasing percentage of positive cells from 48.7 to 75.2%) and H_2O_2 (increasing percentage of positive cells from 50.4 to 63.5%) (Figs. 3, A and B). To investigate the possible role of ROS in p53 activation by vanadate, the effect of specific modifiers of ROS on vanadate-induced p53 activation was determined. The results show that pretreatment of cells with NAC, catalase, or deferoxamine caused inhibition of vanadate-induced p53 activation ($p < 0.05$) (Fig. 4), whereas increasing H_2O_2 generation with the addition of SOD or NADPH enhanced p53 activation ($p < 0.05$) (Fig. 4). These effects on vanadate-induced p53 activation are consistent with the effects on ROS generation (Fig. 2). These data support the hypothesis that ROS generation by vanadate is required for its activation of p53. It is noted that treatment of cell with sodium formate not only did not inhibit vanadate-induced p53 activation but also enhanced vanadate-induced p53 activation (Fig. 4).

Induction of Apoptosis by Vanadate—p53 is believed to be crucial in the induction of apoptosis in human and murine cells following DNA damage (5, 6, 8). This notion was supported by the findings that apoptosis of both thymocytes and intestinal crypt cells following irradiation was blocked in p53-deficient mice (5, 6, 37, 38). To study the molecular basis of vanadate-induced apoptosis, we established an apoptosis model using JB6 Cl 41 cells. The results from DNA fragmentation assay showed that treatment of cells with vanadate indeed caused a dose-dependent induction of apoptosis in Cl 41 cells (Figs. 5A). Flow cytometric analysis of DNA fragment end labeling indicated that apoptosis increased by 11.6, 38.2, and 51.8% at the concentrations of 50, 100, and 400 μ M, respectively (Fig. 5B), whereas the control group only had 3.0% positive cells. It was noted that apoptosis induction at 400 μ M vanadate was higher than that at 200 μ M, whereas p53 activation at 400 μ M is less than that at 200 μ M. The explanation for this is that there were more apoptotic cells at 400 μ M than at 200 μ M, which resulted in less p53 activity observed at

400 μ M. Exposure of the human lung cell line (A549) to 200 μ M vanadate also increased apoptosis by 35.6% (Fig. 5C). These results suggest that vanadate-induced apoptosis is not only limited in mouse epidermal Cl 41 cells.

Induction of Mitochondrial Damage by Vanadate—Mitochondrial damage is a key step for apoptosis in many experimental systems (32–34). Changes of mitochondrial transmembrane potential ($\Delta\psi_m$) have been considered an indicator of mitochondrial damage (32–34). JC-1 and DiOC₆ are two dyes widely used for determination of $\Delta\psi_m$ (32–34). We employed these dyes and found that treatment of cells with vanadate resulted in a significant decrease in $\Delta\psi_m$ as measured by JC-1 staining (from 61.6 to 19.3%) and DiOC₆ staining (from 38.9 to 6.0%) (Fig. 6). These data indicate that vanadate causes mitochondrial membrane damage.

ROS-mediated p53 Activation Plays an Essential Role in Mitochondrial Damage and Apoptosis by Vanadate—To study the ROS-mediated p53 activation in vanadate-induced mitochondrial damage, the cells were preincubated with various ROS modifiers for 30 min, and then the cells were used to study mitochondrial damage in response to vanadate. As shown in Fig. 7, the effects of these ROS modifiers on $\Delta\psi_m$ changes are in agreement with their effects on vanadate-induced p53 activation (Fig. 7).

To obtain direct evidence for the involvement of ROS-mediated p53 activation in vanadate-induced apoptosis, we used two fibroblast cell lines, p53^{+/+} and p53^{-/-}, which were derived from mouse embryos containing either wild-type p53 (p53^{+/+}) or were p53-deficient (p53^{-/-}) as reported previously (5, 6, 31). p53^{+/+} fibroblasts exhibited increases of apoptosis by 41.6, 36.6, and 25.7% at vanadate doses of 800, 200, or 50 μ M, respectively, whereas p53^{-/-} cells showed very weak responses (16.0, 9.0, and 0.0% at vanadate doses of 800, 200, or 50 μ M, respectively) (Fig. 8). These results demonstrate that p53 activation mediated by H_2O_2 is required for vanadate-induced apoptosis. We also observed that there are some apoptotic cells in p53-deficient cells, revealing that there may be some other pathways involved in vanadate-induced apoptosis.

DISCUSSION

We reported previously that vanadate can generate ROS, which are considered to be involved in apoptosis induction (24). However, the molecular mechanisms of apoptosis caused by vanadate-generated ROS remain to be investigated. The results presented in this study demonstrate that ROS generated by vanadate mediate p53 activation and mitochondrial damage, which subsequently leads to cell apoptosis. This conclusion is based on the observations that exposure of cells to vanadate resulted in activation of p53 activity, generation of ROS, and a decrease in mitochondrial transmembrane potential as well as cell apoptosis. Reduction of vanadate-induced H_2O_2 by catalase, NAC, or deferoxamine inhibited the p53 activation and cell mitochondrial damage induced by vanadate. In contrast, increasing H_2O_2 generation with SOD or NADPH promoted p53 activation and mitochondrial damage. Furthermore, vanadate-induced apoptosis occurred at a much higher level in cells expressing wild-type p53 (p53^{+/+}) than in p53-deficient (p53^{-/-}) cells.

It is well accepted that extracellular stimuli trigger signals through a cascade of protein-protein interactions (1–5, 39–41). It is generally believed that these extracellular stimuli generate and/or require reactive free radicals or derived oxidant species to transmit successfully their signals to the nucleus (40, 42). Naturally occurring free radicals typically include ROS and reactive nitrogen species (40). In addition to inducing cellular injury, such as DNA damage and lipid peroxidation, free radicals also function as intracellular messengers (19, 40, 43).

FIG. 3. Determination of O_2^- and H_2O_2 by HE and DCFH-DA staining. Cl 41 cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with vanadate 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. *a* and *c* are HE and DCFH-DA controls, respectively; *b* and *d* are cells with 200 μ M vanadate treatment.

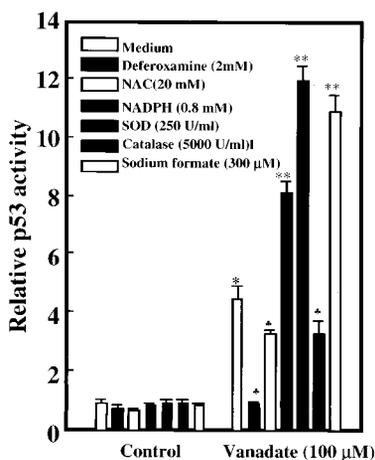
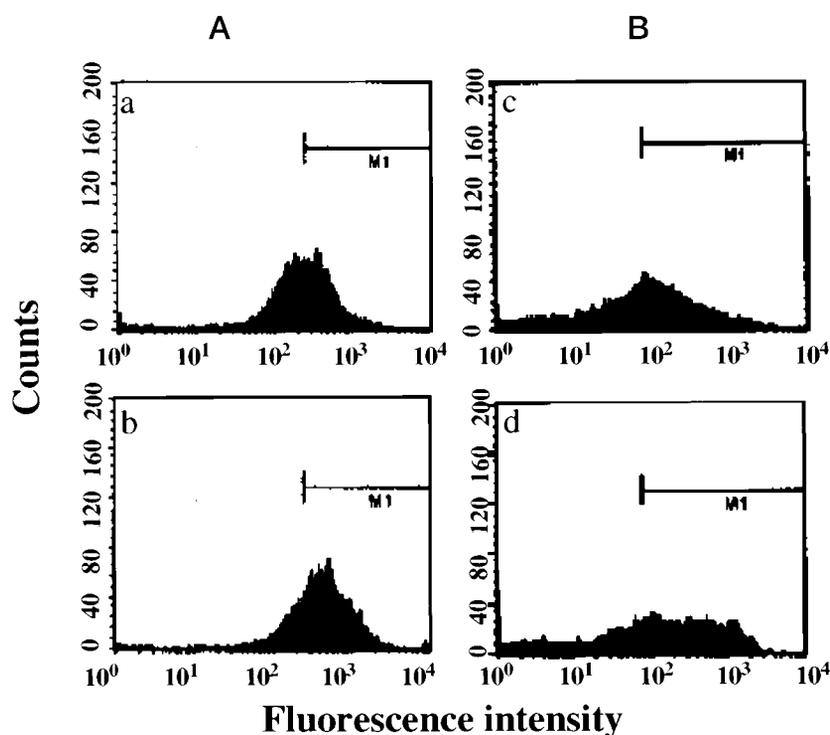


FIG. 4. Effects of free radical scavengers on p53 activation by vanadate. JB6, Cl 41, and PG13-luciferase (PG13-luc) stable transfectant (Cl 41 p53) cells suspended in 5% FBS/MEM were added to each well of 96-well plates and cultured overnight. The cells were pretreated with different free radical modifiers at the concentrations indicated. The cells were then exposed to vanadate (100 μ M) for 24 h. The p53 activity was determined by the luciferase activity assay. The results are presented as relative p53 activity. Each column and bar indicates the mean \pm S.D. from triplicate assays. The * indicates a significant increase from control; \clubsuit indicates a significant decrease from vanadate; and ** indicates a significant increase from vanadate ($p < 0.05$).

More and more data are accumulating to indicate a vital role of ROS in mediating cellular responses by various extracellular stimuli (19, 24, 26, 32, 40, 43). It has been reported that free radicals are involved in the production of cytokines, growth factors, and hormones in the activation of nuclear transcription factors, in gene transcription, in neuromodulation, and in apoptosis (19, 40, 43). For example, it has been reported that generation of H_2O_2 is required for platelet-derived growth factor signal transduction (44). The evidence suggesting the involvement of ROS in apoptosis includes the following: 1) the addition of ROS or deletion of endogenous antioxidants can induce apoptosis; 2) apoptosis can be inhibited by endogenous

or exogenous antioxidants in some cases; and 3) apoptosis is associated with increases in cellular ROS levels (45). Our previous studies have indicated that vanadate can generate ROS, which are considered to be involved in apoptosis induction (24). The results presented here demonstrate that increased intracellular H_2O_2 levels and activation of p53 activity were detected upon incubation of cells with vanadate. Pretreatment of cells with NAC or catalase prevented the increase in ROS and resulted in inhibition of p53 activation by vanadate. In contrast, increasing H_2O_2 levels with SOD or NADPH led to higher levels of p53 activation. These data suggest that H_2O_2 plays an essential role in vanadate-induced p53 activation. Although the details of molecular mechanism for involvement of H_2O_2 in p53 activation by vanadate are not clear, it is reasonable to hypothesize that H_2O_2 -mediated DNA damage and activation of other signal transduction pathways, such as mitogen-activated protein kinase family, may cause an increased p53 protein expression and p53 protein phosphorylation, respectively. It should be noted that pretreatment of cells with sodium formate enhanced p53 activation by vanadate. These data are consistent with our previous finding that sodium formate promotes vanadate-induced apoptosis, supporting our notion that \cdot OH is not the positive regulator for p53 activation by vanadate. The explanation for enhancing effects of vanadate-induced p53 activation by sodium formate may be due to other pathways by which \cdot OH feedback down-regulates p53 activation. Our next study will focus on this issue.

Alteration of mitochondrial function has been linked to cell apoptosis in most cases. An inhibition of oxidative ATP production has been reported to be associated with glucocorticoid-induced lymphocyte apoptosis (46). A decrease in the ability of mitochondrial dehydrogenase to cleave tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) has been demonstrated in anti-CD3-induced apoptosis of T cells (33). In the case of tumor necrosis factor- α -induced apoptosis, early disruption of mitochondrial function has also been described (34). Detailed study has indicated that alterations of mitochondrial functions include an early decrease in $\Delta\psi_m$, a drop in the rate of mitochondrial translation and defect in

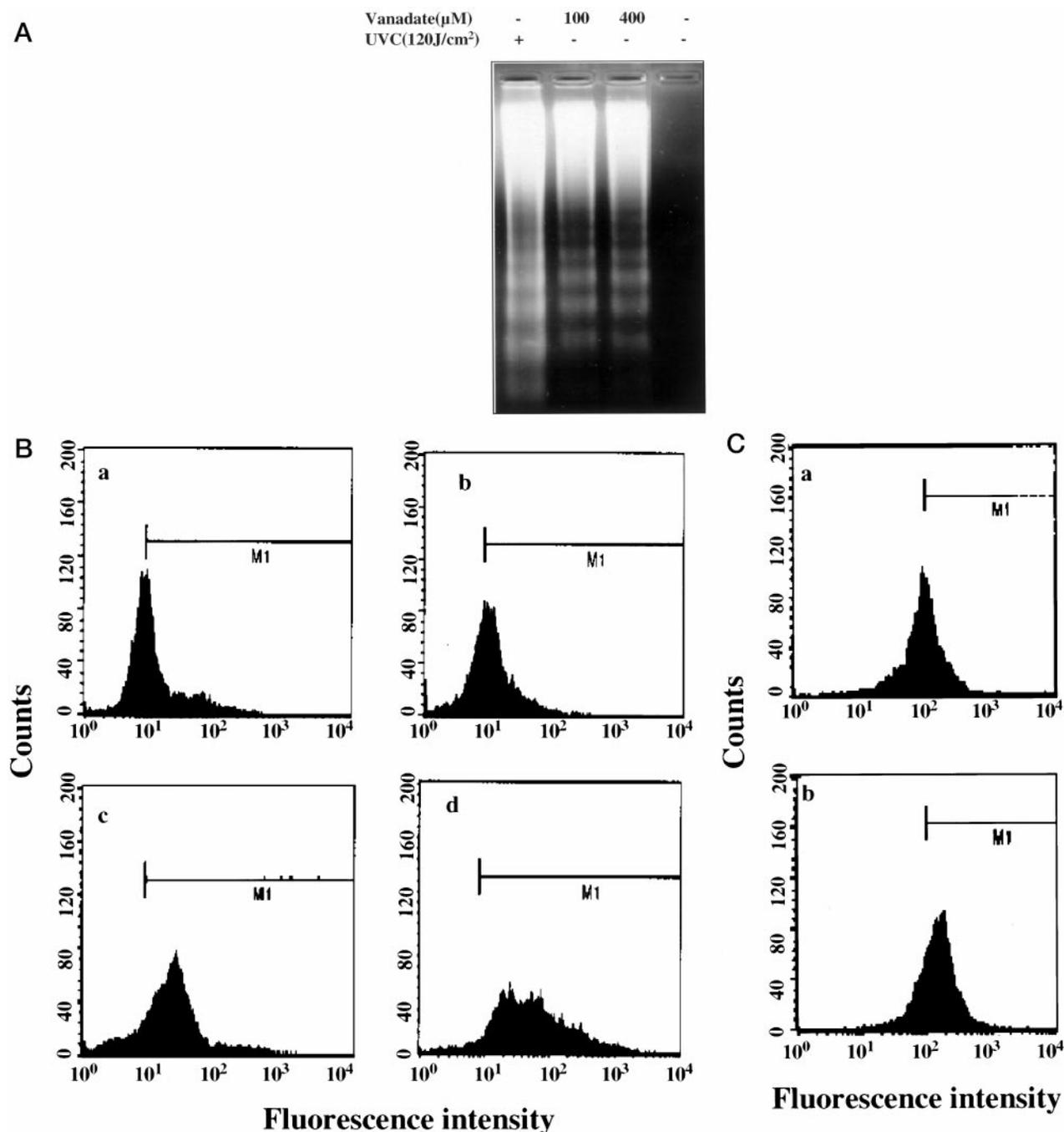


FIG. 5. Induction of apoptosis by vanadate. *A* and *B*, subconfluent (80–90%) monolayers of JB6 Cl 41 cells in 100-mm dishes were subjected to either ultraviolet C (UVC) ($120\text{ J}/\text{m}^2$, positive control) or different concentrations of vanadate for 30 h. Then both detached and attached cells were harvested. *A*, DNA fragmentation assays were carried out as described under “Materials and Methods”; *B*, DNA fragment end labeling assays were performed as described using an *in situ* cell death detection kit employing fluorescein (Roche Molecular Biochemicals). The cells were analyzed by flow cytometry. The concentrations of vanadate used were as follows: *a*, medium control; *b*, $50\ \mu\text{M}$; *c*, $100\ \mu\text{M}$; and *d*, $400\ \mu\text{M}$. *C*, A549 cells were treated with vanadate for 30 h. The cells were harvested, and DNA fragment end labeling assays were carried out using the same method described in *B*. The concentrations of vanadate used were as follows: *a*, medium control; *b*, $200\ \mu\text{M}$ vanadate.

mitochondrial protein cytoplasmic precursor maturation (47). Among all of these changes, the decrease in $\Delta\psi_m$ may be most tightly associated with cell apoptosis. Indeed, overexpression of Bcl-2 or treatment of cells with ionophore nigericin resulted in both an increase in $\Delta\psi_m$ and the inhibition of apoptosis (48). The data from the current investigation demonstrate a drop in $\Delta\psi_m$ after exposure of cells to vanadate. This alteration of mitochondrial function could be blocked by either scavenging H_2O_2 or deletion of the p53 gene. These data therefore indicate that generation of ROS is required for vanadate-induced mitochondrial damage.

Apoptosis is a naturally occurring process of cell “suicide” that plays a critical role in the development and maintenance of metazoans by eliminating superfluous or unwanted cells (1–4, 11–12). Disruption of apoptosis plays a major role in diseases such as cancer, AIDS, autoimmune disease, and neurodegeneration (11–18). The biochemical machinery for apoptotic cell death is constitutively present in virtually all mammalian cells and can be activated by a wide variety of extra- or intracellular signals (1–6, 24). Although numerous investigations have been dedicated to the elucidation of apoptosis initiation and regulation, fundamental questions concerning the

FIG. 6. Mitochondrial damage induced by vanadate. Cl 41 cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with vanadate for 12 h. The dye JC-1 (A) or DiOC₆ (B) dissolved in Me₂SO and diluted with PBS to final concentrations of 10 μ g/ml or 40 nM, respectively, 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. *a* and *c* are JC-1 and DiOC₆ controls, respectively, and *b* and *d* are cells treated with 200 μ M vanadate.

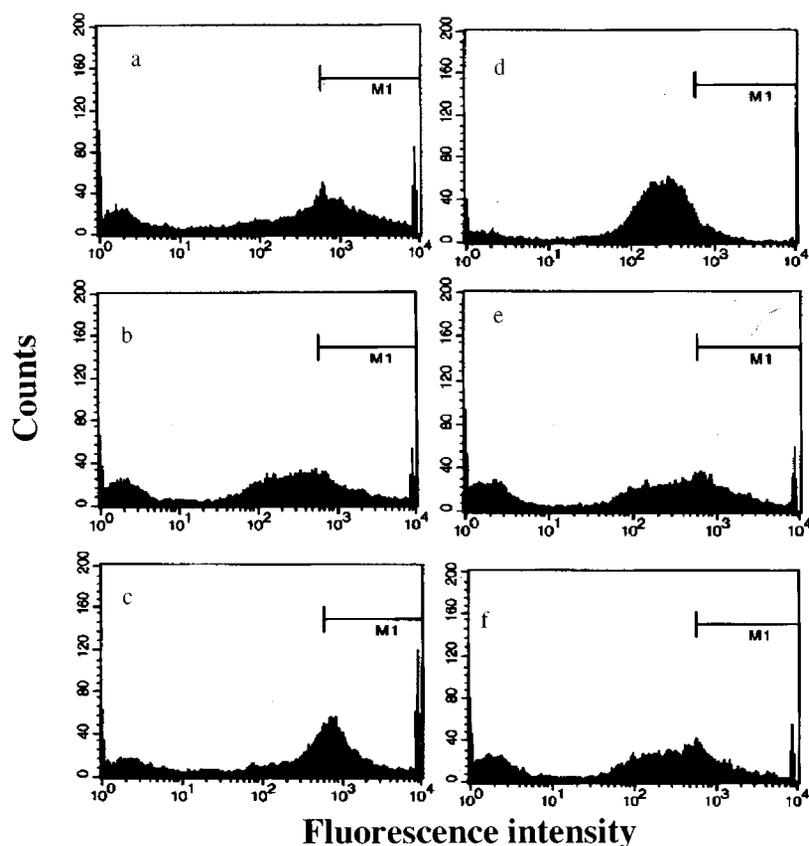
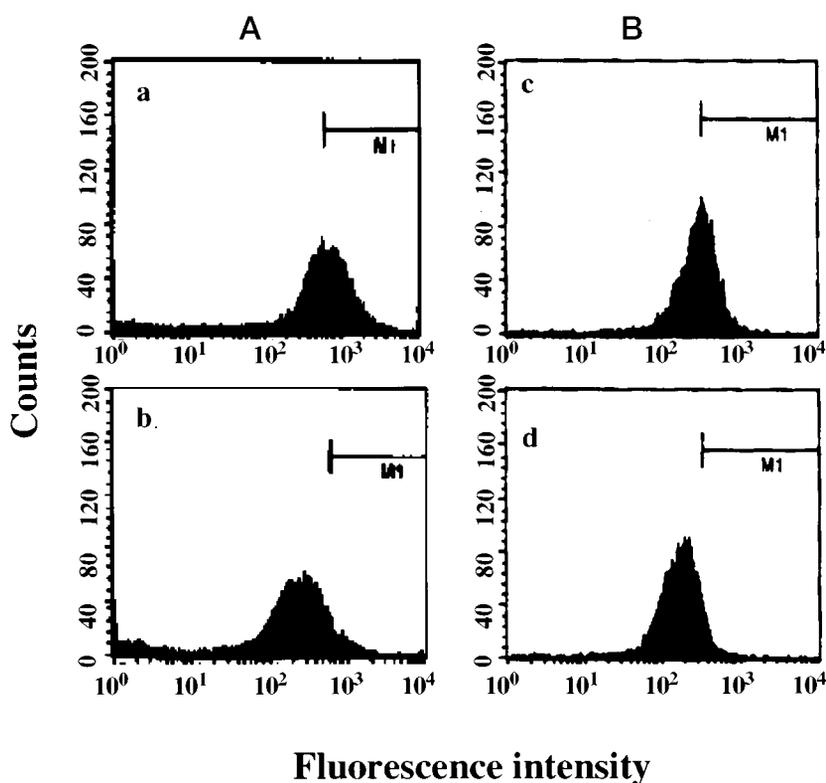


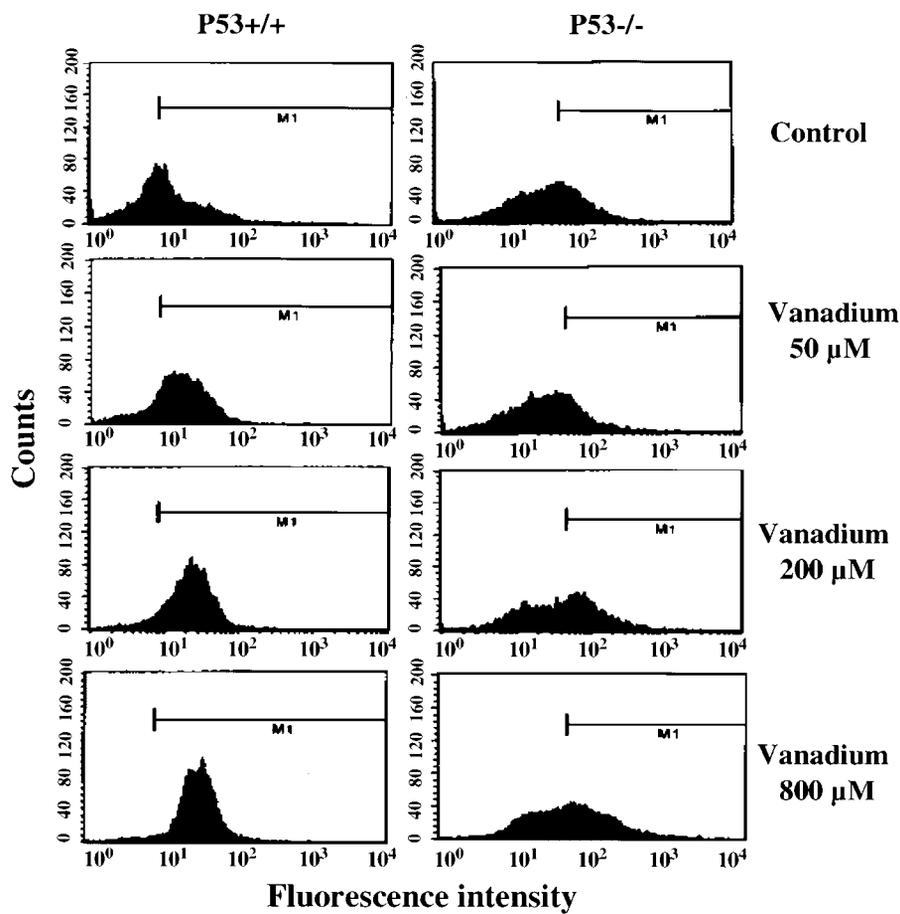
FIG. 7. Effects of free radical modifiers on the decrease in mitochondrial transmembrane potential induced by vanadate. Cl 41 cells suspended in 5% FBS/MEM were added to each well of 6-well plates and cultured overnight. The cells were pretreated with different free radical modifiers as indicated for 30 min. The final concentrations of these modifiers are as follows: catalase, 5×10^4 units/ml; SOD, 250 units/ml; sodium formate, 300 μ M; or deferoxamine, 2 mM. The cells were exposed to vanadate at 200 μ M for 12 h. The dye JC-1 was applied to the cells at a final concentration of 10 μ g/ml. The cells were incubated for another 15–20 min at 37 °C, washed twice with PBS, and then harvested for analysis flow cytometry. The percentages of positive cells are as follows: *a*, 40.7% for JC-1 control; *b*, 24.8% for vanadate; *c*, 45.1% for vanadate + deferoxamine; *d*, 8.3% for vanadate + SOD; *e*, 33.6% for vanadate + catalase; and *f*, 23.5% for vanadate + sodium formate.

molecular and biochemical mechanisms of apoptosis elicited by different stimuli remain to be understood. Vanadate has been reported to be an agent with potent toxic effects in a wide variety of experimental systems (21–24). It has been shown to cause DNA mutations and DNA-protein cross-links and apoptosis (21–24). Some previous studies have suggested that va-

nadium-mediated generation of ROS may be involved in toxicity and apoptosis induced by this metal (24–27). In the present investigation, we demonstrated that through H₂O₂-mediated reaction vanadate is able to cause p53 activation, which is required for apoptosis induction by vanadate.

In summary, the results presented in the present study

FIG. 8. Vanadate induces apoptosis in p53+/+ but not in p53-/- fibroblasts. Subconfluent (80–90%) monolayers of p53+/+ or p53-/- fibroblast in 100-mm dishes were subjected to different concentrations of vanadate for 24 h. The cells were then harvested, and DNA fragment end labeling assays were performed as described by using an *in situ* cell death detection kit. The cells were analyzed by flow cytometry.



demonstrate that vanadate induces generation of H_2O_2 , which is required for p53 transactivation. This H_2O_2 -mediated p53 activation appears to play an essential role in vanadate-induced apoptosis.

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