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 H\textsubscript{2}O\textsubscript{2} by N\textsubscript{a}-acetyl-L-cysteine (a general antioxidant) or catalase (a specific H\textsubscript{2}O\textsubscript{2} inhibitor), or the chelation of vanadate by deferoxamine, resulted in inhibition of p53 activation and cell mitochondrial damage. In contrast, an increase in H\textsubscript{2}O\textsubscript{2} 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\textsuperscript{+/-}) but was very weak in p53-deficient (p53\textsuperscript{-/-}) cells. These results demonstrate that vanadate induces p53 activation mainly through H\textsubscript{2}O\textsubscript{2} 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 NH\textsubscript{2}-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)\textsuperscript{1} plays an important role in its adverse biological effects (24–27). Our previous studies also indicate that generation of H\textsubscript{2}O\textsubscript{2} 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; 1 The abbreviations used are: ROS, reactive oxygen species; NAC, N-acetyl-l-cysteine; SOD, superoxide dismutase; DIO\textsubscript{C}, 3,3\textsuperscript{-}dihexyloxacarbocyanine iodide; DCFH-DA, 2',7'-dichlorofluorescin diacetate; HE, dihydroethidium; FBS, fetal bovine serum; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline.

\textsuperscript{¶} To whom correspondence should be addressed: Health Effects Laboratory Division, NIOSH, Morgantown, WV 26505. Tel.: 304-285-6158; Fax: 304-285-5938; E-mail: xas0@cdc.gov.

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Vanadate Induces p53 Transactivation through Hydrogen Peroxide and Causes Apoptosis*
Role of Hydrogen Peroxide in Vanadate-induced p53 Activation

JC-1, DIOC<sub>8</sub>, 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<sup>+ </sup> mouse epidermal cell line (Cl 41) and its stable p53 luciferase reporter plasmid transfected (Cl 41 p53<sup>+</sup> cells) were cultured in monolayers at 37 °C under 5% CO<sub>2</sub> using MEM containing 5% fetal calf serum, 2 mM t-glutamine, and 25 μg/ml gentamicin (28–30). Normal embryo fibroblasts (p53<sup>++/−</sup>) or p53-deficient embryo fibroblasts (p53<sup>−/−</sup>) were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS, 2 mM t-glutamine, and 25 μg/ml gentamicin (31).

Assay for p53 Activity—Confluent monolayers of Cl 41 p53 cells were trypsinized, and 8 × 10<sup>5</sup> 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% CO<sub>2</sub>. 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 (Monolight 2010). 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/m<sup>2</sup>) 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 (ΔΨ<sub>m</sub>) Assay—JC-1 and DIOC<sub>8</sub> 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 DIOC<sub>8</sub> (dissolved in Me<sub>2</sub>SO and diluted with PBS to final concentrations of 10 μM 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 (O<sub>2</sub>·<sup>−</sup>) and H<sub>2</sub>O<sub>2</sub> Staining Assay—HE is a specific O<sub>2</sub>·<sup>−</sup> dye (32), and DCFH-DA has been frequently used to monitor H<sub>2</sub>O<sub>2</sub> 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 Me<sub>2</sub>SO 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 (K<sub>3</sub>CrO<sub>8</sub>) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. Samples were allowed to equilibrate for 30 min at room temperature, and ESR spectra were recorded at 37 °C in a flat cell (Varian E9). Measurements were repeated until consistent signals were observed. The ESR spectra were reproducible.

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 exposure to vanadate (Fig. 1B). These results demonstrated that vanadate is a stimulus for p53 transactivation. It may 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<sub>II</sub> = a<sub>N</sub> = 14.9 G, where a<sub>N</sub> and a<sub>II</sub> denote hyperfine splittings of the nitroxyl nitrogens and α-hy-
hydrogen, 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 ‘OH radical generation. Addition of catalase, a scavenger of \( \text{H}_2\text{O}_2 \), inhibited ‘OH radical generation (Fig. 2C), indicating that \( \text{H}_2\text{O}_2 \) was produced in the vanadate-treated cells and served as a precursor of ‘OH generation. Addition of sodium formate, an ‘OH radical scavenger, decreased the signal intensity (Fig. 2D), confirming that the 1:2:2:1 quartet observed in this study was due to ‘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 \( \text{O}_2^- \) or DCBH-DA, a fluorescent dye for \( \text{H}_2\text{O}_2 \), demonstrate that incubation of cells with vanadate led to an increase in the generation of both \( \text{O}_2^- \) (increasing percentage of positive cells from 48.7 to 75.2%) and \( \text{H}_2\text{O}_2 \) (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 \( \text{H}_2\text{O}_2 \) 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, 5B). 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 \( \mu \text{M} \), respectively (Fig. 5B), whereas the control group only had 3.0% positive cells. It was noted that apoptosis induction at 400 \( \mu \text{M} \) vanadate was higher than that at 200 \( \mu \text{M} \), whereas p53 activation at 400 \( \mu \text{M} \) is less than that at 200 \( \mu \text{M} \). The explanation for this is that there were more apoptotic cells at 400 \( \mu \text{M} \) than at 200 \( \mu \text{M} \), which resulted in less p53 activity observed at 400 \( \mu \text{M} \). Exposure of the human lung cell line (A549) to 200 \( \mu \text{M} \) 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 (\( \Delta \phi_{\text{m}} \)) have been considered an indicator of mitochondrial damage (32–34). JC-1 and DiOC2 are two dyes widely used for determination of \( \Delta \phi_{\text{m}} \) (32–34). We employed these dyes and found that treatment of cells with vanadate resulted in a significant decrease in \( \Delta \phi_{\text{m}} \) as measured by JC-1 staining (from 61.6 to 19.3%) and DiOC2 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 \( \Delta \phi_{\text{m}} \) 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 \( \mu \text{M} \), respectively, whereas p53−/− cells showed very weak responses (16.0, 9.0, and 0.0% at vanadate doses of 800, 200, or 50 \( \mu \text{M} \), respectively) (Fig. 8). These results demonstrate that p53 activation mediated by \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) by catalase, NAC, or deferoxamine inhibited the p53 activation and cell mitochondrial damage induced by vanadate. In contrast, increasing \( \text{H}_2\text{O}_2 \) 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).
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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) levels with SOD or NADPH led to higher levels of p53 activation. These data suggest that \( \text{H}_2\text{O}_2 \) plays an essential role in vanadate-induced p53 activation. Although the details of molecular mechanism for involvement of \( \text{H}_2\text{O}_2 \) in p53 activation by vanadate are not clear, it is reasonable to hypothesize that \( \text{H}_2\text{O}_2 \)-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 \( \text{OH} \) 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 \( \text{OH} \) 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-\( \alpha \)-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 \( \Delta\psi_{\text{m}} \), a drop in the rate of mitochondrial translation and defect in
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 \( \text{H}_2\text{O}_2 \) 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
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 vanadium-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 H$_2$O$_2$-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...
Role of Hydrogen Peroxide in Vanadate-induced p53 Activation

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 H2O2, which is required for p53 transactivation. This H2O2-mediated p53 activation appears to play an essential role in vanadate-induced apoptosis.

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