Molecular and Cellular Biochemistry **202:** 9–17, 1999.

Vanadate induces apoptosis in epidermal JB6 P^+ cells via hydrogen peroxide-mediated reactions

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Received 25 January 1999; accepted 5 May 1999

Abstract

Apoptosis is a physiological mechanism for the control of DNA integrity in mammalian cells. Vanadium induces both DNA damage and apoptosis. It is suggested that vanadium-induced apoptosis serves to eliminate DNA-damaged cells. This study is designed to clarify a role of reactive oxygen species in the mechanism of apoptosis induced by vanadium. We established apoptosis model with murine epidermal JB6 P^+ cells in the response to vanadium stimulation. Apoptosis was detected by a cell death ELISA assay and morphological analysis. The result shows that apoptosis induced by vanadate is dose-dependent, reaching its saturation level at a concentration of 100 μM vanadate. Vanadyl (IV) can also induce apoptosis albeit with lesser potency. A role of reactive oxygen species was analyzed by multiple reagents including specific scavengers of different reactive oxygen species. The result shows that vanadate-induced apoptosis is enhanced by NADPH, superoxide dismutase and sodium formate, but was inhibited by catalase and deferoxamine. Cells exposed to vanadium consume more molecular oxygen and at the same time, produce more H_2O_2 as measured by the change in fluorescence of scopoletin in the presence of horseradish peroxidase. This change in oxygen consumption and H_2O_2 production is enhanced by NADPH. Taken together, these results show that vanadate induces apoptosis in epidermal cells and H_2O_2 induced by vanadate plays a major role in this process. (Mol Cell Biochem **202**: 9–17, 1999)

Key words: cellular apoptosis, vanadium, hydrogen peroxide, reactive oxygen species

Introduction

Vanadium is an essential trace element in plants and animals. It is widely distributed in rocks, soil, and to a lesser extent in water [1–3]. This metal regulates growth factor mediated signal transduction pathways, promotes cell transformation and decreases cell adhesion [4–6]. Among various oxidation states of vanadium, the pentavalent state is the most stable form. At physiological pH, vanadium (V) is found as vanadate anion [4]. This ion has a host of biological activities, with the most important one being an analogous effect as phosphate in biological systems, thus inhibiting the enzymes mediating phosphate transfer reactions such as ATPases and kinases [7]. Vanadate-containing compounds exert potent toxic effects on a wide variety of biological systems [4, 8–12]. Vanadium compounds were reported to modify DNA synthesis [14–16], cause direct DNA damage and induce DNA strand breaks [17]. Epidemiological studies have shown a correlation between vanadium exposure and the incidence of lung cancer in humans, but no correlation between vanadium exposure and skin cancer has been established [18, 19].

Apoptosis is a term used to describe individual cells in the process of programmed cell death that undergoes a distinct set of morphological changes. Apoptosis can be induced by

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a variety of stimuli, including depletion of growth factors, hormones, heat shock, γ-irradiation, and cross-linking of Fas antigen [20]. In a disease such as cancer, there is imbalance between the rates of cell division and cell death [21, 22]. Any chemical agent that promotes or suppresses apoptosis can alter this balance, resulting in neoplasia [22]. The skin cells of a human body have a high exposure rate to vanadium contained in the residual oil fly ash, which is emitted by power plants and other industries that burn heavy oil [23]. Why vanadium can not induce skin cancer? To address the question, we used murine epidermal JB6 cells as an experimental model to investigate the mechanism involved in the process.

While the mechanisms of apoptosis are not yet completely understood, the involvement of reactive oxygen species (ROS) has been suggested [24, 25]. It may be noted that vanadate-mediated generation of ROS are believed to play an important role in the mechanism of cellular damage caused by this metal [17, 27–29]. For example, in the presence of NADPH, several flavoenzymes, such as glutathione reductase, reduce vanadate to vanadium (IV). During the reduction process, molecular oxygen is reduced to $O2^{-}$ radical and then to H_2O_2. The vanadium (IV) reacts with H_2O_2 to produce ·OH radical via a Fenton-like reaction ($V(IV) + H_2O_2 \rightarrow V(V) + {\cdot}OH + OH^{-}$) [27]. This radical is able to cause DNA damage, including hydroxylation of dG residues in DNA to generate 8-hydroxy-2′-deoxyguanosine [17]. Thus, it is likely that vanadate may induce cellular responses through ROS. In the present study, the following questions will be addressed: (a) Does vanadate induce apoptosis in epidermal cells? (b) If yes, does ROS play a role? (c) Which species among these ROS plays the most critical role in vanadate-induced apoptosis?

Materials and methods

Reagents

Sodium metavanadate (vanadium (V), $NaVO_3$, or vanadate) and vanadyl sulfate trihydrate (Vanadium (IV), $VOSO_4$, vanadyl) were purchased from Aldrich (Milwaukee, WI, USA). β-nicotinamide adenine dinucleotide phosphate (NADPH), deferoxamine and N-acetyl-L-cysteine were purchased from Sigma (St. Louis, MO, USA). Superoxide dismutase (SOD) and catalase were purchased from Boehringer Mannheim (Indianapolis, USA). Sodium formate was purchased from Fisher (Pittsburgh, PA, USA). All these reagents were freshly made in phosphate-buffered solution (pH 7.4).

Cell culture

The murin epidermal JB6 P^+ cells have been widely used as an experimental model in carcinogenesis studies. It can be easily transformed into tumor cells by a variety of chemicals. Therefore, it is a good model for evaluating the carcinogenic effect of vanadate on the skin cells. The cells were cultured at 37°C in a 5% CO_2 incubator with a complete culture medium composed of Eagle's minimal essential medium (EMEM) that was supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells form a monolayer after confluence. Trypsin (0.25%) EDTA solution was used to detach the cells from the culture flask for splitting and passing the cells. Vanadate or vanadyl was dissolved in PBS and used at concentrations as described in the text or figure legends.

Apoptosis assay

ELISA assay

The JB6 P^+ cells were plated in a 96 well plate at a density of 1×10^4 cells/well 1–24 h before the cells were subjected to treatment in triplicate wells. After treatment, the cells were washed twice in phosphate-buffered solution and apoptosis was quantitated by measuring the levels of cytosolic histone-bound DNA fragments (a cell death ELISA assay kit; Boehringer Mannheim, Indianapolis, USA). The assays were carried out according to the protocol provided by the manufacturer. Briefly, the cells were lysed with 200 μl of lysis buffer at room temperature. The lysate from three identical wells was combined and 20 μl of the resulting lysate was mixed with 80 μl of antibody solution in the coated wells. The loaded wells were incubated at room temperature for 2 h. The substrate was added to each well after it was washed three times in washing buffer. After incubation at 37 °C for 10–20 min, the reaction was stopped and optical density was measured using a microplate reader with a light filter of 405 nm. The readings were used to represent the degree of apoptosis. Vanadate-induced apoptosis was expressed as the mean value of three separate experiments.

Morphological analysis

The vanadium-induced apoptosis was also examined by morphological analysis. At the end of cell treatment, the cells were washed twice in phosphate-buffered solution and then fixed with 10% buffered formalin. The fixed cells were stained with Wright staining kit containing solutions A, B and C (Volu-Sol Inc., Louisville, KY, USA). The staining was conducted in the 96 well tissue culture plates according to the protocol provided by the manufacturer. The cells were stained in 100 μl Wright's stain solution (solution A) for 2 min, treated with 100 μl phosphate-buffered solution (solution B) for 1.5 min, and then rinsed in solution C for 10 sec. After being air-dried, the stained cells were observed under a Leitz inverted microscope. The cell image was captured with a Sony 3CCD color video camera and printed out with a Sony color video printer.

Oxygen consumption measurements

Oxygen consumption measurements were carried out using a Gilson Oxygraph equipped with a Clark microelectrode, model 516 (Gilson Medical Electronics, Middleton, WI, USA). Cells (1.0×10^6) were suspended in 1 ml phosphate-buffered solution and pre-incubated at 37°C for 10 min. The basal oxygen consumption or vanadate-induced oxygen consumption was monitored over a period of 10 min at 37°C in the absence or presence of vanadate (40 μM).

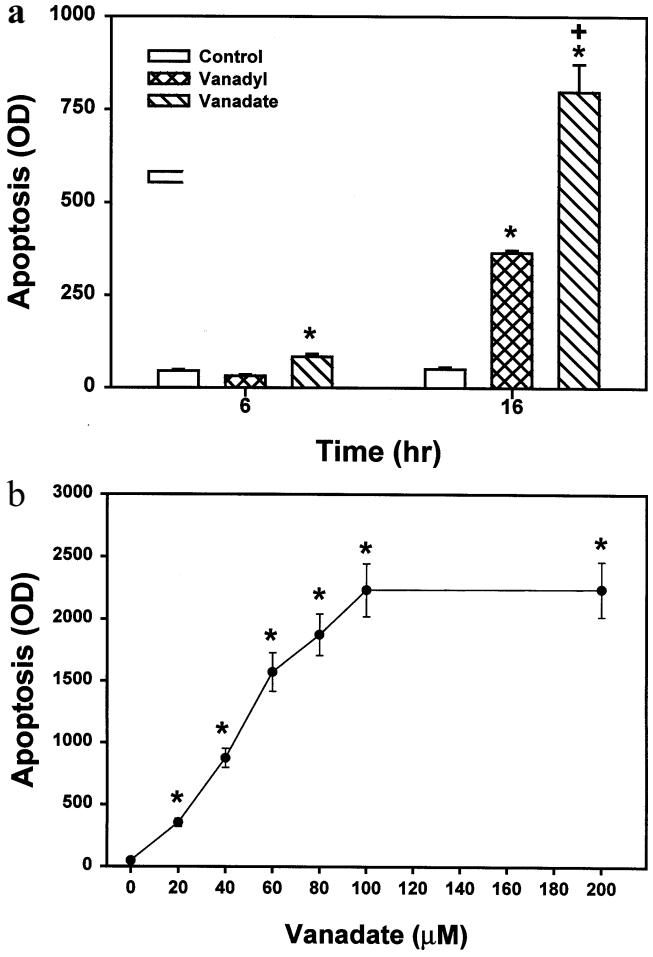

Fig. 1. Vanadium-induced apoptosis in JB6 P^+ cells. The cells were plated in a 96 well plate at a density of 1×10^4 cells/well in 200 μl of the complete culture medium for 24 h before vanadium treatment. Each bar indicates the mean and S.D. of three assay wells from 3 independent experiments. *indicates a significant increase in apoptosis from control ($p < 0.05$). (A) Time effect: Vanadate or vanadyl was added to the incubation mixture at a final concentration of 40 μM. After incubation for 6 or 16 h, the cells were washed twice in PBS (pH 7.4) and then subjected to the cell death ELISA assay. The plus indicate a significant increase in apoptosis from vanadyl ($p < 0.001$); (b) Dose-dependence of vanadate-induced apoptosis. Apoptosis was examined with ELISA assay after 16 h exposure to vanadate.

H_2O_2 measurements

H_2O_2 was measured by monitoring the change in fluorescence of scopoletin in the presence of horseradish peroxidase. Cells (1.0×10^6) were suspended in 1 ml phosphate-buffered solution. Fluorescence was monitored at an excitation wavelength of 350 mn and an emission wavelength of 460 nm using a Perkin-Elmer fluorescence spectrophotometer (model MPG-36).

Data analysis

Data that are reported as mean ± S.D. of results from 3 individual experiments was analyzed by the Student's *t*-test at a confidence level $p < 0.05$–0.001.

Oxygen consumption measurements

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Results

Vanadium induces apoptosis in JB6 epidermal cells

JB6 cells were exposed to vanadate or vanadyl at a concentration of 40 μM for 6 or 16 h. Apoptosis was monitored using a cell death ELISA assay kit (Fig. 1a). The results showed that after the 6 h exposure, only vanadate induced apoptosis. After the 16 h exposure, both vanadate and vanadyl were able to induce apoptosis, but vanadate exhibited much greater potency than vanadyl. Effects of vanadate concentration in the range of 5–200 μM were examined in a 16 h exposure condition (Fig. 1B). A significant increase in apoptosis was observed at concentrations of 20 μM and above, reaching a plateau at 100 μM. Morphological changes in the vanadate-treated cells were also observed (Fig. 2). In the presence of 20 μM vanadate, the cells started to shrink and form long, narrow, cytoplasmic processes (Fig. 2B). As the concentration of vanadate increased to 40 μM, these morphological changes became more distinct (Fig. 2C), and finally at 100 μM vanadate, many cells contracted and turned into small condensed cells (Fig. 2D). In the process of condensing, some of the cells retained short cytoplasmic processes, while others lost this characteristic and became round. With a higher magnification, condensation of the nucleus was clearly observed in the vanadate-treated cells (Fig. 3).

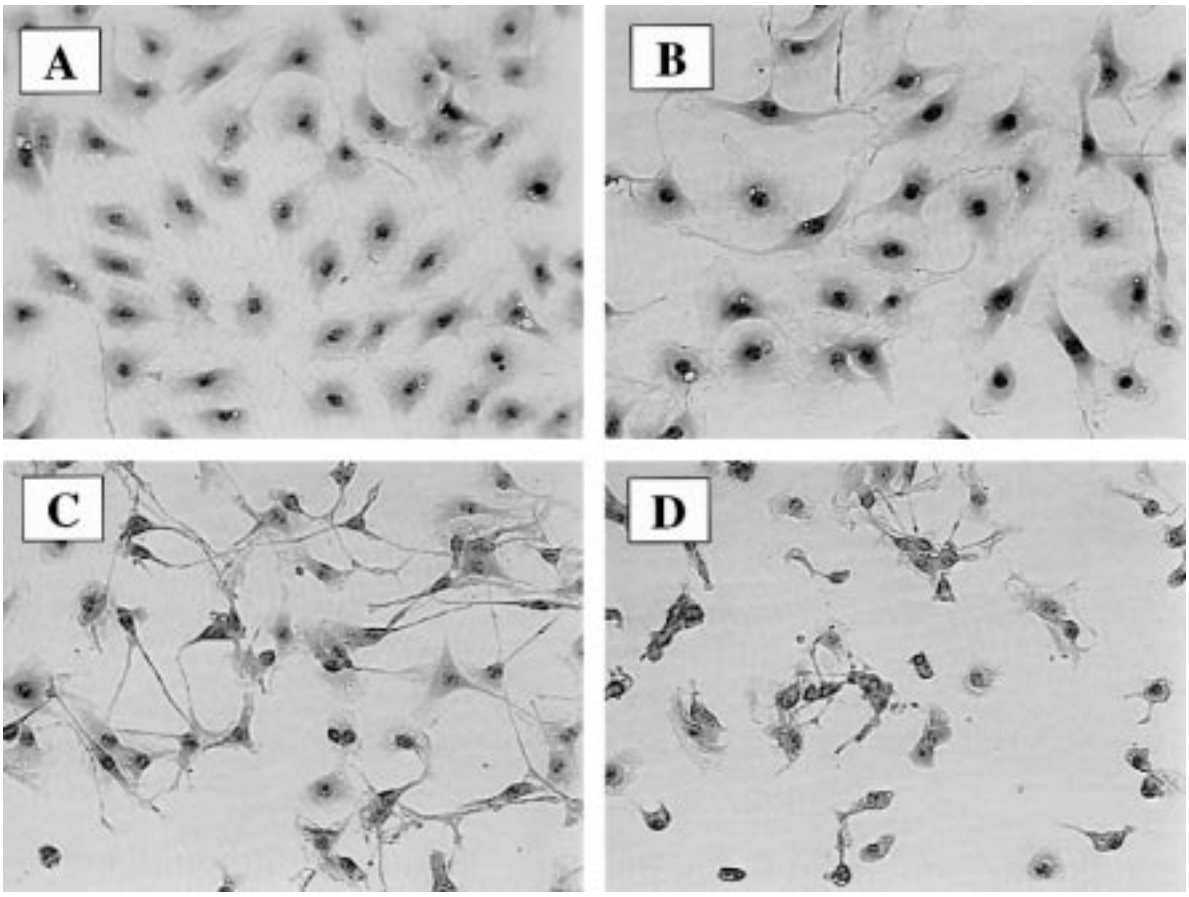

Fig. 2. Morphological changes of JB6 P^+ cells induced by vanadate. The vanadate concentrations were (A) non; (B) 20 μM; (C) 40 μM; (D) 100 μM vanadate. Exposure time was 16 h. Magnification: 400 ×.

NADPH enhanced vanadate-induced apoptosis

Cellular reduction of vanadate generates vanadium (IV) and ROS. This reaction is enhanced by NADPH. The role of ROS in vanadate-induced apoptosis was examined by a combined treatment of the cells with NADPH and vanadate (Fig. 4). Addition of NADPH enhanced apoptosis by 5-fold. NADPH alone did not exhibit a significant activity in the induction of apoptosis. Microscopic evaluation reveals dramatic morphological changes in cells treated with vanadate plus NADPH for 16 h (Fig. 5). This result suggests that ROS is involved in the initiation of apoptosis by vanadate.

Catalase inhibits vanadate-induced apoptosis

Catalase, a scavenger of H_2O_2, was used to evaluate the role of H_2O_2 in vanadate-induced apoptosis (Fig. 6). A combination of vanadate and NADPH was used to induce apoptosis in this experiment. Addition of catalase inhibited vanadate-induced apoptosis by 75%. Accordingly, catalase also mitigated the morphological changes resulting from the treatment of vanadate plus NADPH (data not shows). This result indicates a role of H_2O_2 in the molecular mechanisms of apoptosis induced by vanadate.

Deferoxamine inhibits vanadate-induced apoptosis

Deferoxamine has been reported to decrease vanadate-induced oxidant generation and related DNA damage [30, 31]. In the present study, deferoxamine was added to examine its possible protection against vanadate-induced apoptosis (Fig. 7). Deferoxamine was added at a concentration of 1 mM in the presence of vanadate and NADPH. Indeed, deferoxamine provided significant protection against apoptosis. Morphologically, 95% of the cells treated by vanadate plus NADPH remained in their original shape in the presence of deferoxamine (data not shown).

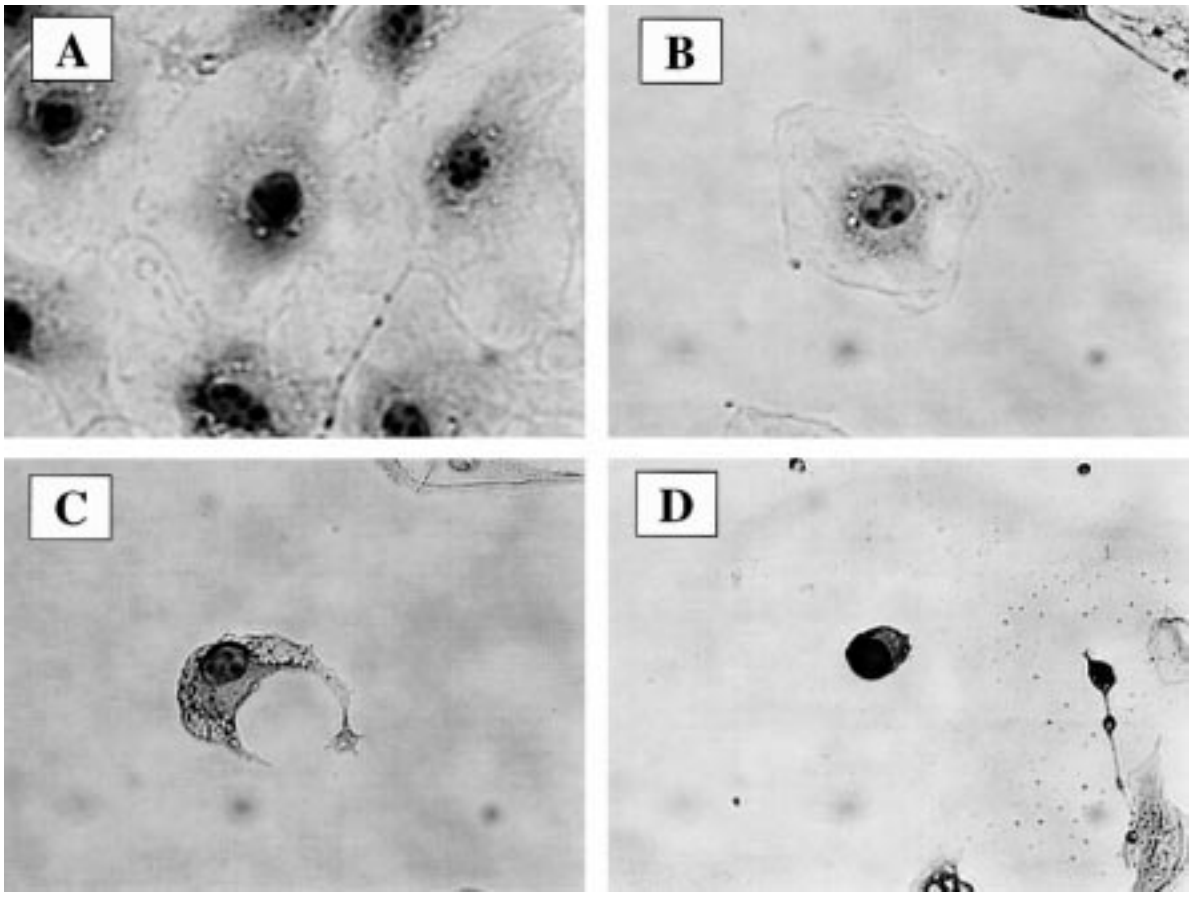

Fig. 3. Nuclear condensation induced by vanadate. (A) Cells without vanadate; (B), (C) and (D) Cells after 16 h exposure to vanadate. Three different stages of nuclear condensation in cells treated by 80 μM vanadate. Magnification: 630 ×.

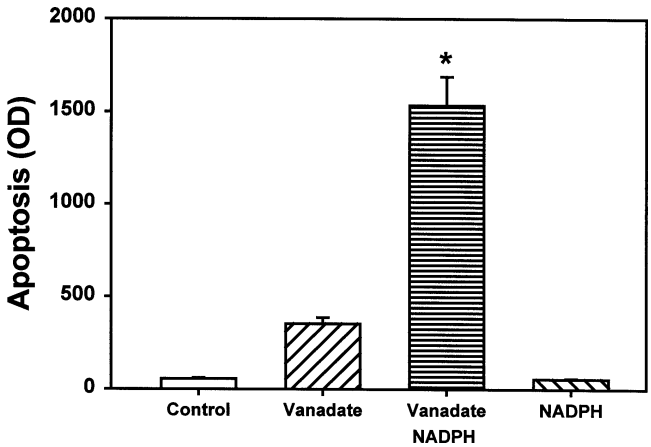

Fig. 4. Effect of NADPH on apoptosis induced by 20 μM vanadate. Experimental conditions were the same as those in Fig. 1. NADPH was added to cells at a final concentration of 0.5 mM. Values are means ± S.D. of 3 experiments. *indicates a significant increase in apoptosis from cells treated by vanadate alone ($p < 0.001$).

Sodium formate enhances vanadate-induced apoptosis

As an hydroxyl radical (·OH) scavenger, sodium formate, was used to evaluate the role of ·OH radical in vanadate-induced apoptosis (Fig. 8). Sodium formate enhanced vanadate-induced apoptosis. This ·OH radical scavenger itself did not cause any significant apoptosis. The enhancement of vanadate-induced apoptosis by sodium formate was also demonstrated morphologically (picture not shown).

Superoxide dismutase enhances vanadate-induced apoptosis

Superoxide dismutase (SOD), whose function is to catalyze the dismutation of superoxide radical to generate H_2O_2, was used to examine the role of $O2^-$ in the vanadate-induced apoptosis (Fig. 9). The result showed that SOD enhances the vanadate-induced apoptosis. SOD alone did not cause any apoptosis.

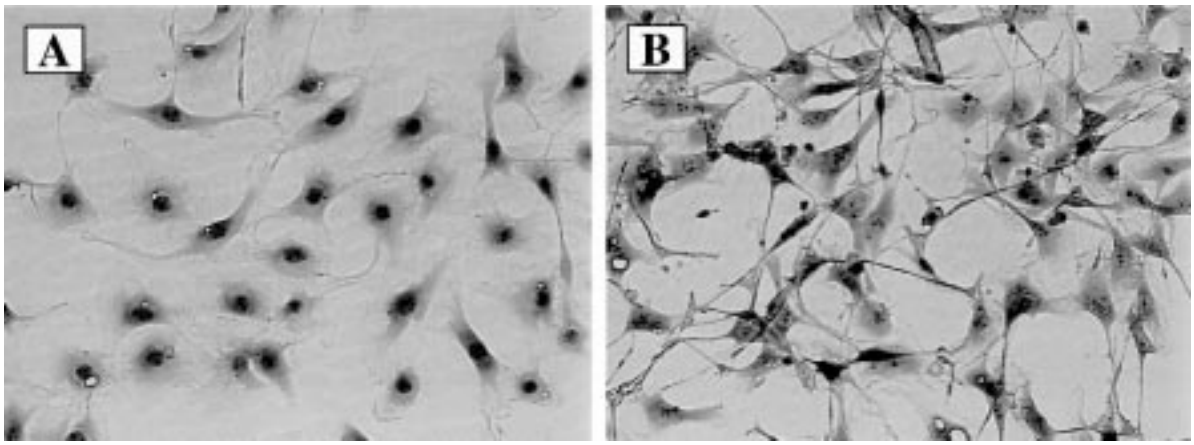

Fig. 5. Morphological change of JB6 P′ cells induced by 20 μM vanadate. (A) 20 μM vanadate; (b) 20 μM vanadate plus 0.5 mM NADPH. Note the increased formation of long process. Magnification: 400 ×.

Vanadate enhances oxygen consumption and H_2O_2 release in JB6 cells

Generation of ROS is usually associated with an increase in oxygen consumption in the cells and an enhancement in H_2O_2 release from the cells. To confirm the generation of ROS in the vanadate treated cells, oxygen consumption and H_2O_2 production were examined (Fig. 10). Figure 10A shows results of oxygen consumption measurements. Cells alone consumed molecular oxygen at a steady basal rate (1147 ± 134 nM/10^6 cells/h), while vanadate or vanadate plus NADPH enhanced the oxygen consumption rate by 40 and 106% respectively. Figure 10B shows H_2O_2 release by vanadate-stimulated cells. Cells alone released H_2O_2 at a low basal level (1.35 ± 0.25 pM/10^6 cells). Vanadate-stimulated cells released a significantly higher level (86% more) of H_2O_2. Addition of NADPH further enhanced the vanadate-stimulated H_2O_2 release by 62 fold.

Discussion

The results obtained in the present study show that vanadate is able to induce apoptosis in epidermal JB6 P^+ cells. Among the ROS examined, H_2O_2 is the one responsible for vanadate-

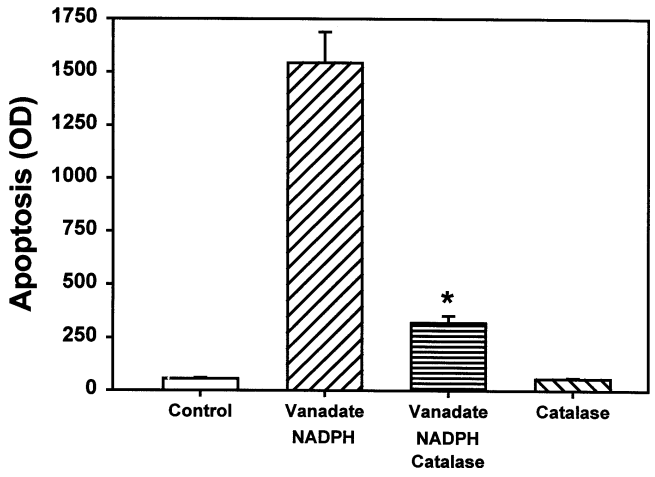

Fig. 6. Effect of catalase on vanadate-induced apoptosis. Cells were treated with 20 μM vanadate plus 0.5 mM NADPH for 16 h to induce apoptosis. Catalase was added to the cells at 5000 unit/ml 30 min before vanadate exposure. Experimental conditions were the same as those in Fig. 1. Values are means ± S.D. of 3 experiments. *indicates a significant decrease in apoptosis from cells treated by vanadate plus NADPH ($p < 0.001$).

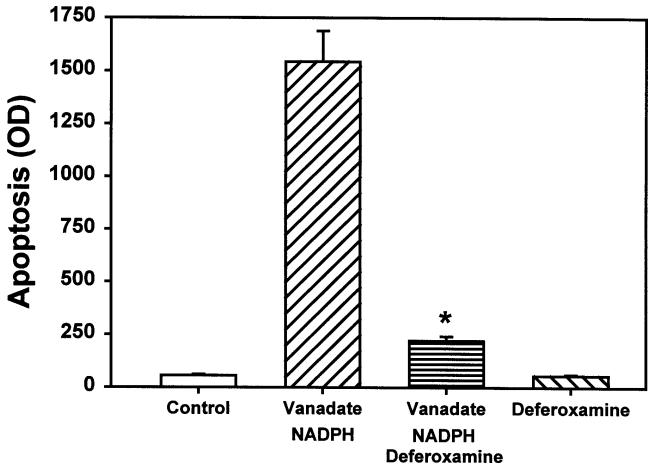

Fig. 7. Effect of deferoxamine on vanadate-induced apoptosis. The same condition as in Fig. 6 was used to examine the effect of deferoxamine (1 mM). Values are means ± S.D. of 3 experiments. *indicates a significant decrease in apoptosis from cells treated by vanadate plus NADPH ($p < 0.001$).

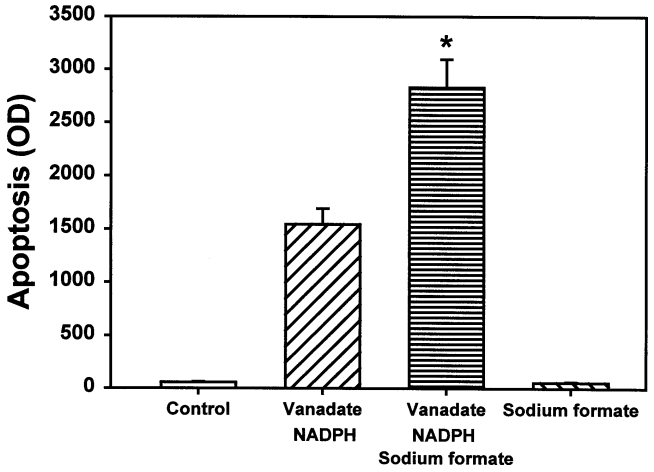

Fig. 8. Effect of sodium formate on vanadate-induced apoptosis: The same condition as in Fig. 6 was used to examine the effect of sodium formate (5 mM). Values are means ± S.D. of 3 experiments.

induced apoptosis. The following experimental observations support this conclusion: (a) It has been reported that cellular reduction of vanadate generates vanadium (IV) and ROS [27, 29]. The major vanadate reductants include certain flavoenzymes, such as glutathione reductase. NAD(P)H is required as a co-factor in the enzymatic reduction of vanadate. As shown in the present study, NADPH enhanced vanadate-induced apoptosis by 5-fold; (b) A metal chelator, deferoxamine, inhibited vanadate-apoptosis. This chelator renders vanadate less reducible toward its lower oxidation state and decreases the generation of ROS; (c) Catalase, a specific scavenger of H_2O_2, inhibited vanadate-induced

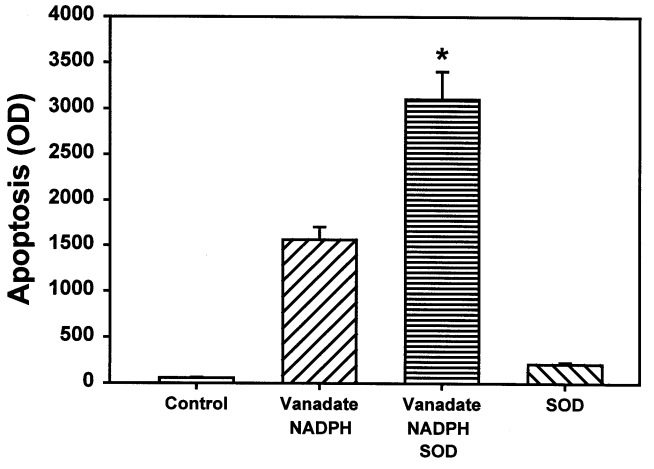

Fig. 9. Effect of superoxide dismutase on vanadate-induced apoptosis. The same condition as in Fig. 6 was used to examine effect of superoxide dismutase (800 unit/ml). Values are means ± S.D. of 3 experiments. *indicates a significant increase in apoptosis from cells treated by vanadate plus NADPH ($p < 0.001$).

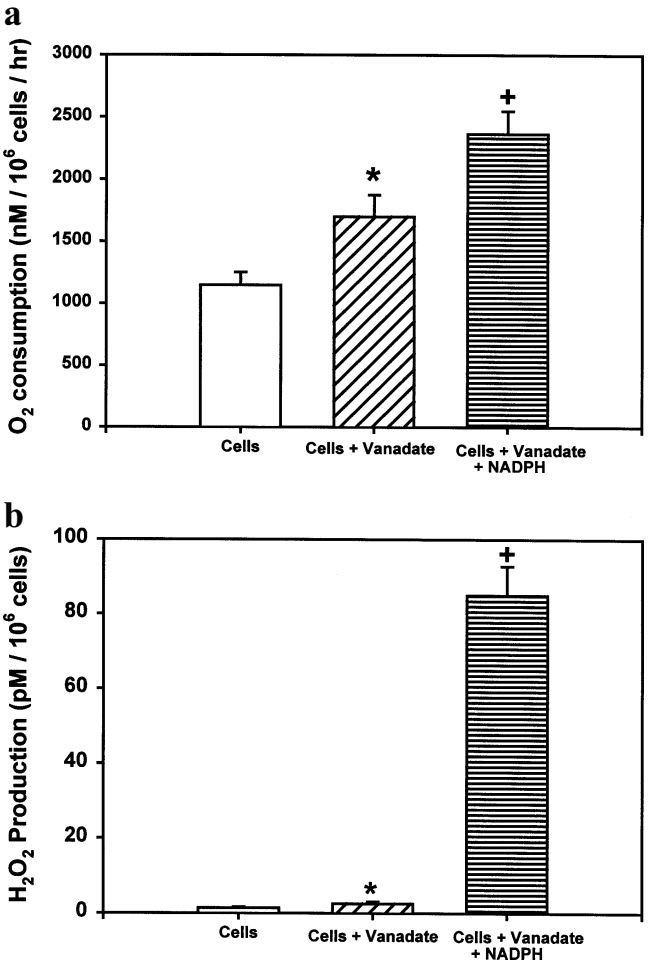

Fig. 10. Oxygen consumption and H_2O_2 release in vanadate treated cells. The assay was carried out in PBS (pH 7.4). Cells (1.0×10^6) were used in each reaction. The concentration of vanadate and NADPH were 40 μM and 0.5 mM respectively. * or $^+$ indicate a significance increase from control or vanadate alone ($p < 0.001$). (A) Oxygen consumption: Oxygen consumption rate is expressed as nM per 1×10^6 cells/h; (B) H_2O_2 production: H_2O_2 production rate is expressed as pM per 1×10^6 cells.

apoptosis; (d) SOD enhanced this apoptosis by catalyzing the dismutation of O_2^- to generate more H_2O_2. The enhancement effect of SOD indicates that O_2^- itself did not play a significant role in vanadate-induced apoptosis and supports the role of H_2O_2; (e) H_2O_2 itself has been reported to induce apoptosis [32]. We observed the same effect of H_2O_2 on the epidermal cells in this study (data not shown); (f) Vanadate stimulated cells to release H_2O_2 which was generated by the reduction of molecular oxygen. Inside the cells, the level of H_2O_2 is regulated by SOD, catalase and glutathione peroxidase. It has been reported that exposure of cells to an inhibitor of glutathione peroxidase or catalase reduced not only cell proliferation but also apoptosis. SOD reduced cell proliferation and enhanced apoptosis [32].

Sodium formate enhanced vanadate-induced apoptosis. Sodium formate is an ·OH radical scavenger. ·OH radical is considered as a common messenger for NF-κB activation. Recent studies [33, 34] have shown that activation of Nf-κB reduces the signal for cell death and protects cells from apoptosis. It is possible that sodium formate inhibited NF-κB activation and thus enhanced the apoptosis via scavenging ·OH. Further studies are needed to understand the role of ·OH.

Inability to remove excess or unwanted cells by apoptosis has been implicated in a number of disorders [20–23, 35]. Apoptosis is thought to be a physiological form of programmed cell death in which a cell is triggered to die by external stimuli [36], thereby provoking its own demise through the implementation of an endogenous 'suicide' mechanism [37]. Because of the fundamental importance of apoptosis in the regulation of tissue growth, alteration in this pathway may be important in carcinogenesis [21]. This hypothesis was supported by alterations of expression of a number of oncogenes, tumor suppressor genes and cell cycle regulatory genes that are associated with either apoptosis or inappropriate cell survival leading to tumor formation. While the signal transduction pathway that triggers apoptosis is still not fully elucidated, a number of proximate induction conditions have been identified, including growth factor and tumor necrosis factor α. Recent studies have shown that a variety of DNA damaging agents induce apoptosis [21]. These agents include ultraviolet B light [38], ionizing radiation [38], vanadyl [39] and chromate [40]. Interestingly, all of these DNA damaging agents are able to induce generation of reactive oxygen species. It is possible that these agents and vanadate may cause apoptosis via a common mechanism, i.e., ROS mediated reactions as a common mechanism.

Vanadate-containing compounds exert potent toxic and carcinogenic effects on a wide variety of biological systems [4–13]. A correlation between vanadium exposure and the 12 incidence of lung cancer in humans has been found. The skin cells may use the apoptosis as a protective mechanism against vanadium-induced cellular damage. The damaged cells which escape apoptosis may be responsible for vanadate-induced carcinogenesis. The vanadate-induced apoptosis could play a passive role in elimination of these genetic lesions. Imbalance between proliferation and apoptosis must be established for neoplastic growth to occur, therefore investigation of vanadate-induced apoptosis in the skin cells will improve our understanding of the overall mechanism of vanadate-induced carcinogenesis.

In summary, the results obtained in the present study yield the following conclusions: (a) Vanadate is able to cause apoptosis in JB6 cells; (b) Vanadyl is also able to induce apoptosis in these cells albeit to a lower degree; (c) NADPH enhances the vanadate-mediated generation of ROS and enhances vanadate-induced apoptosis; (d) The inhibitory effect of catalase provides strong evidence that H_2O_2 plays an important role in vanadate-induced apoptosis; (e) Deferoxamine inhibits the ability of vanadate to generate ROS and apoptosis; (f) SOD enhances vanadate-induced apoptosis via enhancement of H_2O_2-generation, again supporting the role of H_2O_2 in the vanadate-induced apoptosis; (g) H_2O_2 was generated by the reduction of molecular oxygen via O_2^- as an intermediate; (h) It is possible that formate, an ·OH radical scavenger, enhanced vanadate-induced apoptosis through inhibition of NF-κB activation.

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