Available online at www.sciencedirect.com
ScienceDirect
Toxicology and Applied Pharmacology 220 (2007) 83–91
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Phosphorylation of p53 at serine 15 in A549 pulmonary epithelial cells exposed to vanadate: Involvement of ATM pathway

Katsura Suzuki, Kiyoshi Inageda, Gen Nishitai, Masato Matsuoka *

Department of Hygiene and Public Health I, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

Received 16 September 2006; revised 23 December 2006; accepted 28 December 2006
Available online 8 January 2007

Abstract

When A549 cells were exposed to sodium metavanadate ($NaVO_3$), the pentavalent species of vanadium (vanadate), phosphorylation of p53 protein at Ser15 was found in a time (8–48 h)- and dose (10–200 μM)-dependent manner. After the incubation with 50 or 100 μM $NaVO_3$ for 48 h, accumulation of p53 protein was accompanied with Ser15 phosphorylation. Among serines in p53 protein immunoprecipitated from A549 cells treated with 100 μM $NaVO_3$ for 48 h, only Ser15 was markedly phosphorylated. Treatment with other vanadate compounds, sodium orthovanadate (Na_3VO_4) and ammonium metavanadate (NH_4VO_3), also induced Ser15 phosphorylation and accumulation of p53 protein. While phosphorylation of extracellular signal-regulated protein kinase (ERK) was found in cells treated with $NaVO_3$, treatment with U0126 did not suppress Ser15 phosphorylation. On the other hand, treatment with wortmannin or caffeine, the inhibitors to phosphatidylinositol 3-kinase related kinases (PIKKs), suppressed both $NaVO_3$-induced Ser15 phosphorylation and accumulation of p53 protein. The silencing of ataxia telangiectasia mutated (ATM) expression using short-interference RNA resulted in the marked suppression of Ser15 phosphorylation in A549 cells exposed to $NaVO_3$. However, treatment with antioxidants such as catalase and *N*-acetylcysteine did not suppress $NaVO_3$-induced Ser15 phosphorylation. Transcriptional activation of p53 and DNA fragmentation in A549 cells treated with $NaVO_3$ were suppressed only slightly by S15A mutation, suggesting that Ser15 phosphorylation is not essential for these responses. The present results showed that vanadate induces the phosphorylation of p53 at Ser15 depending on ATM, one of the members of PIKK family, in this human pulmonary epithelial cell line.

Keywords: Vanadate; p53; Ser15 phosphorylation; ERK; ATM; A549

Introduction

Epidemiological studies have established that elevations in the concentration of ambient air particulate matter (PM) are associated with lung cancer and cardiopulmonary mortality (Brunekreef and Holgate, 2002; Pope et al., 2002). Residual oil fly ash (ROFA), the fine particles generated from the combustion of fuel oil, is a complex chemical mixture components including a transitional metal vanadium (Ghio et al., 2002). Due to the high proportion of vanadium in the fine particles with an aerodynamic mass median diameter ≤2.5 μm ($PM_{2.5}$), a considerable fraction of vanadium seems to reach the alveoli where this metal contacts with the lung epithelium directly (Sørensen et al., 2005). In mice and rats exposed to vanadium pentoxide by whole-body inhalation, alveolar/bronchiolar neoplasms, and respiratory tract proliferative and inflammatory lesions were observed (Ress et al., 2003). Although vanadium compounds interfere with mitosis and chromosome distribution, and induce DNA strand brakes (Barceloux, 1999), the molecular mechanisms of vanadium-induced pulmonary cell damage, and of its repair have not been clarified.

The tumor suppressor p53 protein functions primarily as a transcriptional factor, and plays an important role in the control of genomic integrity, or the elimination of damaged or

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ATM-Rad3-related protein; DMSO, dimethyl sulfoxide; DNA-PK, DNA-activated protein kinase; DSB, DNA double-strand break; ERK, extracellular signal-regulated protein kinase; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PIKK, phosphatidylinositol 3-kinase related kinase; siRNA, short-interference RNA.

* Corresponding author. Fax: +81 3 5269 7419.
E-mail address: matsuoka@research.twmu.ac.jp (M. Matsuoka).

0041-008X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2006.12.028

tumorigenic cells (Bargonetti and Manfredi, 2002; Sengupta and Harris, 2005). In response to a variety of cellular stresses, p53 protein is phosphorylated on multiple residues in both the amino- and carboxy-terminal domains by several different protein kinases (Bode and Dong, 2004). Among serine residues, phosphorylation at Ser15 has been shown to be responsible for the stabilization, subsequent induction and transactivation function of p53 (Dumaz and Meek, 1999; Shieh et al., 1997; Siliciano et al., 1997). In addition, phosphorylation of mouse p53 at Ser18 (corresponding to Ser15 of human p53) is required for the maximum p53-mediated response to DNA damage (Chao et al., 2000). It has been reported that treatment with vanadium compound induces the transactivation of p53 activity in Cl 41 mouse epidermal cell line (Huang et al., 2000), and the upregulation of p21 expression and G_2/M phase arrest in A549 human type II alveolar epithelial cell line (Zhang et al., 2001, 2003). In addition, increased immunopositivity of p53 protein was found in the liver of rats given vanadium compound (Chakraborty et al., 2005). To our knowledge, however, it has not been studied whether or not vanadium induces the phosphorylation of p53 in the lung epithelial cells.

Vanadium exists in oxidation states ranging from −1 to +5, preferentially +3 (vanadium), +4 (vanadyl) and +5 (vanadate). Among them, vanadate is known to be the most toxic (Barceloux, 1999). In the present study, we examined the effects of vanadate exposure on the phosphorylation of p53 at Ser15 and other serine residues in A549 cells which express wild-type p53 (Jia et al., 1997). Using inhibitors to the members of serine/threonine kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase related kinase (PIKK), we determined the protein kinase responsible for vanadate-induced Ser15 phosphorylation. Thereafter, using short-interference RNA (siRNA), the effects of knockdown of ataxia telangiectasia mutated (ATM), a member of PIKK family (Shiloh, 2003), on Ser15 phosphorylation was examined. Since it has been reported that A549 cells generate reactive oxygen species in response to vanadate exposure (Zhang et al., 2001, 2003), effects of antioxidants on vanadate-induced phosphorylation of p53 at Ser15 were also examined. Finally, in order to elucidate the biological significance of vanadate-induced Ser15 phosphorylation, p53–DNA binding activity and DNA fragmentation were determined in A549 cells introduced a missense mutation of p53 cDNA plasmid that changed serine 15 to alanine (S15A).

Materials and methods

Chemicals. Sodium metavanadate ($NaVO_3$) and ammonium metavanadate (NH_4VO_3) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and sodium orthovanadate (Na_3VO_4), caffeine and *N*-acetylcysteine were from Sigma Chemical Co. (St. Louis, MO). U0126, wortmannin, catalase and hydrogen peroxide were purchased from Calbiochem (La Jolla, CA). Phospho-p53 (Ser6, Ser9, Ser15, Ser20, Ser37, Ser46 and Ser392) antibodies, phospho-p44/42 MAPK (ERK) (Thr202/Tyr204) antibody, and phosphorylation state-independent ERK antibody were obtained from Cell Signaling Technology, Inc. (Beverly, MA). p53 (DO-1 and Pab 1801) antibodies, p53 (Pab 1801) antibody agarose conjugate, actin (I-19) antibody, and Atm (2C1) antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Cell Count Reagent SF was purchased from Nacalai Tesque (Kyoto, Japan).

Cell culture and treatments. A549 cells were obtained from Health Science Research Resources Bank (Japan Health Sciences Foundation, Osaka, Japan), and grown in Earle's Minimum Essential Medium with non-essential amino acids, 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO, Invitrogen Corp., Carlsband, CA) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. For each experiment, exponentially growing A549 cells were plated at 5×10^5 cells/well in 6-well culture plates, 6×10^4 cells/well in 24-well culture plates, 2×10^4 cells/well in 96-well culture plates, or 4×10^6 cells/flask in 75 cm^2 culture flasks, and cultured for 1 day before the experiments. Vanadate compounds were dissolved in water and sterilized by filtration. U0126 and wortmannin were dissolved in dimethyl sulfoxide (DMSO). Caffeine and catalase were dissolved in distilled water. *N*-Acetylcysteine was dissolved in PBS immediately before use, and the pH was adjusted to 7.4 with 2N NaOH. A549 cells were preincubated with serum-free medium containing each compound for 30 min (for wortmannin and caffeine) or 1 h (for U0126 and catalase). For the treatment with *N*-acetylcysteine, cells were preincubated with complete medium containing it for 12 h, and washed with PBS before the exposure to $NaVO_3$. Untreated control cells were preincubated with medium alone or containing the same concentration of DMSO (0.2% for U0126 and 0.002% for wortmannin). Then, preincubated and control cells were treated with or without $NaVO_3$ for 48 h at 37 °C.

siRNA transfection. Duplexed siRNA targeted against the human *ATM* gene (ATM1 pub. siRNA) was obtained from Qiagen Inc. (Valencia, CA). The sequence of 21-mer siRNA was 5′-GCGCCUGAUUCGAGAUCCUdTdT-3′ (Yoshida et al., 2003). siRNA was transfected into A549 cells grown in 24-well culture plates (50% confluence) using Lipofectamine 2000 (Invitrogen) following the instruction from the manufacturer. After the incubation for 24 h, cells were washed with Earle's Minimum Essential Medium, and kept in serum-free medium for 48 h prior to the experiments.

S15A mutant p53. Total RNA was isolated from A549 cells using Trizol reagent (Invitrogen), and reverse transcription was performed with 0.5 μg of total RNA using the first-strand cDNA synthesis kit (Roche Applied Science, Penzberg, Germany). Full-length p53 cDNA was amplified with polymerase chain reaction (PCR) using primers 5′-CTTAAGCTTGCCACCATGGAGGAGCCGCAGTCAGA-3′ (sense) and 5′-AGGCCTCGAGTCAGTCTGAGTCAGGCCCTTCGT-3′ (antisense), and subcloned into *Hin*dIII/*Xho*I sites in pcDNA3.1 vector (Invitrogen). The nucleotides AGT encoding Ser15 were changed to GCT encoding Ala (S15A) by PCR site-directed mutagenesis. The primers used were 5′-GTCGAGCCCCCTCTGGCTCAGGAAACATTTTCA-3′ (sense) and 5′-TGAAAATGTTTCCTGAGCCAGAGGGGGCTCGAC-3′ (antisense). Mutated site was verified by sequencing. Purified plasmid cDNA (400 ng) with or without S15A mutation was transfected into A549 cells grown in 6-well culture plates (50% confluence) using Lipofectamine 2000. After the incubation for 24 h, cells were washed with medium, and used for the experiments.

Western immunoblotting. After the incubation with vanadate compounds, cells were washed with PBS and lysed with SDS-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected, sonicated and boiled for 5 min. Protein concentration was determined with RC DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein (20 or 30 μg) were subjected to SDS-PAGE on a 6 or 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Buckinghamshire, England). The membrane was blocked with 5% non-fat milk or bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. The membrane was incubated overnight at 4 °C with the primary antibody, and protein was detected with a Phototope-HRP Western blot detection kit (Cell Signaling Technology) or a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Chemical Co., Rockford, IL). After immunodetection, some blots were incubated with Restore Western Blot Stripping Buffer (Pierce Chemical) for 30 min at room temperature, and reprobed with phosphorylation state-independent ERK antibody or actin antibody. The bands on the developed film were quantified with NIH Image Version 1.63 (National Institutes of Health, Bethesda, MD). The density of each band was normalized to that of actin.