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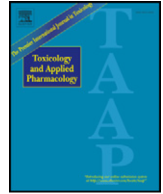

# Vanadium induces dopaminergic neurotoxicity via protein kinase Cdelta dependent oxidative signaling mechanisms: Relevance to etiopathogenesis of Parkinson's disease

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ABSTRACT

Environmental exposure to neurotoxic metals through various sources including exposure to welding fumes has been linked to an increased incidence of Parkinson's disease (PD). Welding fumes contain many different metals including vanadium typically present as particulates containing vanadium pentoxide ($V_2O_5$). However, possible neurotoxic effects of this metal oxide on dopaminergic neuronal cells are not well studied. In the present study, we characterized vanadium-induced oxidative stress-dependent cellular events in cell culture models of PD. $V_2O_5$ was neurotoxic to dopaminergic neuronal cells including primary nigral dopaminergic neurons and the $EC_{50}$ was determined to be 37 μM in N27 dopaminergic neuronal cell model. The neurotoxic effect was accompanied by a time-dependent uptake of vanadium and upregulation of metal transporter proteins Tf and DMT1 in N27 cells. Additionally, vanadium resulted in a threefold increase in reactive oxygen species generation, followed by release of mitochondrial cytochrome *c* into cytoplasm and subsequent activation of caspase-9 (>fourfold) and caspase-3 (>ninefold). Interestingly, vanadium exposure induced proteolytic cleavage of native protein kinase Cdelta (PKCδ, 72–74 kDa) to yield a 41 kDa catalytically active fragment resulting in a persistent increase in PKCδ kinase activity. Co-treatment with pan-caspase inhibitor Z-VAD-FMK significantly blocked vanadium-induced PKCδ proteolytic activation, indicating that caspases mediate PKCδ cleavage. Also, co-treatment with Z-VAD-FMK almost completely inhibited $V_2O_5$-induced DNA fragmentation. Furthermore, PKCδ knockdown using siRNA protected N27 cells from $V_2O_5$-induced apoptotic cell death. Collectively, these results demonstrate that vanadium can exert neurotoxic effects in dopaminergic neuronal cells via caspase-3-dependent PKCδ cleavage, suggesting that metal exposure may promote nigral dopaminergic degeneration.

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## Introduction

Parkinson's disease (PD) is a multifactorial chronic neurodegenerative disorder associated with progressive degeneration of nigral dopaminergic neurons in the mesencephalic midbrain region resulting in substantial loss of dopaminergic neurotransmission to the striatal region (Anglade et al., 1997). The etiopathogenesis of PD is still poorly understood; however, chronic exposure to certain metals such as manganese (Mn) has been implicated in PD pathogenesis (Dobson et al., 2004). Epidemiological and case–control studies conducted in the United States as well as other countries have linked heavy metal exposure to an increased incidence of PD (Gorell et al., 1997; Fleming et al., 1994; Schulte et al., 1996; Liou et al., 1997; Marder et al., 1998; Smargiassi et al., 1998; Taylor et al., 1999; Priyadarshi et al., 2000; Tuchsen and Jensen, 2000; Ritz and Yu, 2000). Subtle preclinical neurological effects have recently been documented following exposure to very low levels of Mn in occupational settings (Mergler, 1999; Crossgrove and Zheng, 2004). Since Mn elimination from the central nervous system (CNS) typically occurs very slowly, delayed neurotoxic effects may occur later in life resulting in an increased frequency of Parkinsonian diseases in the geriatric population (Alessio and Lucchini, 1996; Cranmer et al., 1999; Lucchini et al., 1995, 1997). Early life exposure to heavy metals such as lead has been shown to produce Alzheimer's-like pathology in rodents as well as in primate models (Wu et al., 2008). Two studies have shown that welders are at an increased risk for the development of PD (Racette et al., 2001; Park et al., 2005), while another study did not find such an association between welding and a risk for developing Parkinsonism (Goldman et

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al., 2005; Ellingsen et al., 2008). Welding fumes contain many different metals including manganese, iron, and vanadium, typically present as vanadium pentoxide ($V_2O_5$). However, the neurotoxic effects of $V_2O_5$ are not well understood.

Vanadium continues to be widely used in various industrial applications including steelmaking; arc welding; temperature-resistant alloy production; and glass, pigment and paint manufacturing (Hazardous Substance Database, ChemIDPlus, 2006; Bunting, 2006; McNeilly et al., 2004). Vanadium is a preferred metal for the production of special steels and temperature-resistant alloys because it is one of the lightest high-strength metals. More than 90% of industrial vanadium is used in steel making. The dominant market driver for vanadium over the past three years has been an increased worldwide demand for higher strength steel, most notably in China (Bunting, 2006). This increased demand for vanadium is not expected to decline as the worldwide demand for high quality steel continues. Welding and the associated exposure of workers to welding fumes have increased along with steel production. Among nine metals (Co, Cr, Cu, Fe, Mn, Ni, Ti, V and Zn) characterized in welding fumes by use of inductively coupled plasma mass spectroscopy (ICP-MS), vanadium was present at about half the concentration of Mn (McNeilly et al., 2004). The use of vanadium with non-ferrous metals is of particular importance in the atomic energy industry, aircraft construction and space technology (Hazardous Substance Database, ChemIDPlus, 2006). Notably, vanadium compounds are released into the environment in large quantities, mainly by burning fossil fuels. Vanadium is usually found to be the most abundant trace metal in petroleum samples and can be found in concentrations reaching 1500 mg $kg^{-1}$ depending on the source of the crude oil (Amorim et al., 2007). Vanadium accumulates in the soil, groundwater, and plants that may be consumed by animals and humans (Pyrzynska and Weirzbicki, 2004).

Despite this widespread use of vanadium, the health effects of the metal, in particular the CNS effects, are not well characterized. While earlier studies have shown vanadium exposure in humans may cause CNS depression, tremor, neurasthesia and other severe motor deficits including vegetative symptoms (WHO, 2000; Done, 1979), the neurotoxic effects of vanadium and its potential to induce chronic neurological diseases are not well understood. Another recent study showed that inhaled $V_2O_5$ can damage the nigrostriatal dopaminergic system in rodent models (Avila-Costa et al., 2004), but the mechanism of vanadium-induced dopaminergic neurotoxicity is yet to be defined.

Oxidative stress and apoptosis are regarded as key mediators of neurodegenerative processes in PD (Hartmann et al., 2000; Dawson and Dawson, 1996; Olanow, 2004; Olanow and Tatton, 1999; Olanow, 2004) and are neurotoxic sequelae resulting from metal exposure (Kanthasamy et al., 2003; Kitazawa et al., 2003; Hamai and Bondy, 2004; Latchoumycandane et al., 2005). Our lab previously reported that increased oxidative stress during exposure to Parkinsonian neurotoxicants, as well as pesticides and metals, can activate the proapoptotic kinase PKCδ by caspase-3-dependent proteolysis in cell culture models of PD (Kitazawa et al., 2003; Kaul et al., 2003, 2005a, 2005b; Latchoumycandane et al., 2005). Proteolytic cleavage of PKCδ (74 kDa) by caspase-3 results in a 41 kDa catalytic subunit and a 38 kDa regulatory subunit, leading to a persistent activation of the kinase (Kaul et al., 2003; Kitazawa et al., 2003; Anantharam et al., 2004; Yang et al., 2004). Blockade of proteolytic activation of PKCδ by overexpression of the kinase-dominant negative mutant, cleavage-resistant mutant, or siRNA directed against PKCδ almost completely prevented dopaminergic cell death (Kaul et al., 2003; Kitazawa et al., 2003, 2005; Anantharam et al., 2004; Yang et al., 2004), demonstrating that PKCδ is a key proapoptotic and oxidative stress sensitive kinase in dopaminergic neurons. In the present study, we examined the effect of $V_2O_5$ on oxidative signaling in a dopaminergic cell model of PD.

## Methods

*Chemicals.* Vanadium pentoxide ($V_2O_5$) and MTT were purchased from Sigma (St. Louis, MO); Sytox green nucleic dye and COX-IV antibody were purchased from Molecular Probes (Eugene, OR). Ac-DEVDAFC (Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin), Ac-LEHD-AFC (Acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin), and Z-VAD-FMK (Z-Val-Ala-Asp-fluoromethyl ketone) were purchased from MP Biomedicals (Aurora, OH). Cell Death Detection ELISA plus Assay Kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). RPMI 1640, B27 supplement, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD). Nitric acid was purchased from Fisher Scientific (Pittsburgh, PA). Anti-mouse DMT-1 (1 μg/ml) and anti-mouse transferrin (Tf) (1 μg/ml) were purchased from Alpha Diagnostic International, San Antonio, TX. Protease cocktail, phosphatase inhibitors, ATP, Protein A-Sepharose, protein G-Sepharose and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); rabbit PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); cytochrome *c* antibody was purchased from BD Biosciences, anti-mouse and anti-rabbit secondary antibodies (Alexa Flour 680 conjugated goat anti-mouse IgG, Alexa Flour 680 conjugated donkey anti-goat IgG and Rabbit IgG IR800 Conjugate) were purchased from Invitrogen and Rockland Inc., respectively. [$\gamma$-$^{32}$P]ATP and $^3$H-DA were purchased from Perkin Elmer Life Science (Boston, MA).

*Cell culture.* We used the rat mesencephalic dopaminergic cell line referred to as N27 cells, which was a gift from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO). N27 cells have been used extensively to study the neurotoxic mechanisms pertaining to Parkinson's disease (Clarkson et al., 1999; Kaul et al., 2003, 2005a, 2005b; Miranda et al., 2004; Peng et al., 2005). N27 cells were grown and treated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U of penicillin, and 50 μg/ml of streptomycin in a humidified atmosphere of 5% $CO_2$ at 37 °C as described previously (Kaul et al., 2003; Yang et al., 2004). For antioxidant studies, N27 cells were treated in RPMI 1640 medium supplemented with 2% B27 supplement with or without antioxidants instead of 10% fetal bovine serum.

We also used mouse fetal primary mesencephalic cultures to determine the effect of vanadium on dopaminergic neurons. We prepared nigral primary mesencephalic neuronal cultures from the ventral mesencephalon of gestational 14-d-old mice embryos as described previously (Yang et al., 2004; Zhang et al., 2007). The mesencephalic tissues from mice were dissected, maintained in ice-cold $Ca^{2+}$-free HBSS and then dissociated in HBSS solution containing trypsin-EDTA (0.25%) for 20 min at 37 °C. The dissociated cells were then plated at equal density (0.5 × 106 cells) in 30-mm-diameter tissue culture wells which had been precoated with poly-D-lysine (1 mg/ml). The primary cultures were maintained in a chemically defined medium consisting of neurobasal medium fortified with B-27 supplements, L-glutamine (500 μM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Invitrogen). The cells were maintained in a humidified $CO_2$ incubator (5% $CO_2$, 37 °C) and half of the culture medium was replaced every 2 d. Approximately 5- to 7-d-old cultures were used for experiments.

*Treatment paradigm.* For the purpose of this study, vanadium interchangeably refers to vanadium pentoxide ($V_2O_5$) dissolved in water (moles of $V_2O_5$/l). N27 cells were treated with different concentrations of vanadium for the duration of the experiments using diluted stock solution in culture media. After treatment, cells were collected by trypsinization or scraping, spun down at 200 *g* for 5 min, and washed with ice-cold phosphate-buffered saline (PBS). The lysates from the cell pellets were used for various assays including caspase-3 activity, Western blotting, and measurement of DNA fragmentation.

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