Chapter 2

Vanadium Promotes Hydroxyl Radical Formation By Activated Human Neutrophils

Hypothesis

Vanadium in its different valence states augments the production of reactive oxidants by activated human neutrophils.

Aim

The aim of the experiments described in this chapter was to identify the effects of vanadium in the +2, +3, +4, and +5 valence states on superoxide generation, myeloperoxidase (MPO) activity, and hydroxyl radical formation by activated human neutrophils in vitro, using lucigenin-enhanced chemiluminescence (LECL), autoiodination, and electron spin resonance spectroscopy.

2.1 Introduction

Vanadium is a ubiquitous, naturally occurring, transition metal which is found in high concentrations in the earth's crust, oceans, soil and fossil fuels (Barceloux, 1999b). Metallic vanadium does not exist in nature, but rather as vanadium compounds in oxidation states ranging from -1 to +5, the most common valences being +3, +4 and +5, with quadrivalent salts being the most stable (Barceloux, 1999b). Vanadium is an industrially important metal, which is used primarily in the manufacture of corrosion-resistant metal alloys. Consequently, those who mine and refine vanadium, as well as those involved in the manufacture of metal alloys, especially stainless steel, have high levels of occupational exposure to this metal (Barceloux, 1999b; Vanadium, 2000). Other high-risk occupations include those employed in oil-fired electricity power stations, as well as in the petrochemical industry, because of exposure to vanadium emissions into the atmosphere during the combustion of petroleum, coal and heavy oils (Barceloux, 1999b; Vanadium, 2000; Vanadium, 2001). Adverse health effects associated with excessive exposure to vanadium in the workplace include rhinitis, wheezing, nasal haemorrhage, conjunctivitis, cough, sore throat, and chest pain, all of which are usually transient (Vanadium, 2001), although persistent bronchial hyperresponsiveness and asthma have also been described (Irsigler *et al*, 1999).

Environmental pollution due to vanadium is a potential health threat, albeit of uncertain magnitude (Barceloux, 1999b; Vanadium, 2000; Vanadium, 2001). In the

environmental health setting, vanadium in the atmosphere originates predominantly from the combustion of heavy fuel oils, the metal being a major constituent of residual oil fly ash, which is both persistent and respirable (Van Klaveren & Nemery, 1999). Power plants and other industries that burn heavy oil are the primary offenders, while the contribution of vehicle exhaust fumes is slight because of the low vanadium levels in refined petroleum products. Cigarettes contain vanadium, which is present in both cigarette smoke and ash (Adachi *et al*, 1998) at concentrations comparable with those of iron (Mussalo-Rauhammaa *et al*, 1986).

In both the occupational and environmental settings, exposure to vanadium in the +4 and +5 valence states predominates (Vanadium, 2000) with interconversion between these two valence states occurring in biological systems (Mukherjee *et al*, 2004). Adverse health effects are likely to occur both from metal-induced toxicity and irritant pro-inflammatory effects. In the case of direct toxicity, vanadium compounds are well-recognized inhibitors of different ATPases, particularly Na^+, K^+-ATPase (Sabbioni *et al*, 1991; Mukherjee *et al*, 2004), while the pro-inflammatory activities result from the pro-oxidative activation of several transcription factors, including NFκB, JNK and AP-1, which cooperate to activate genes encoding pro-inflammatory cytokines following exposure of macrophages and T-lymphocytes to the metal *in vitro* (Chen *et al*, 1999; Huang *et al*, 2001). Moreover, inhalation or intracheal instillation of residual oil fly ash, or its major constituent vanadyl sulphate, have been reported to result in up-regulation of genes encoding pro-inflammatory cytokines in rat pulmonary tissue (Chong *et al*, 2000; Nadadur *et al*, 2002).

The neutrophil, a prototype inflammatory cell which is poorly responsive to conventional anti-inflammatory chemotherapy (Thomson *et al*, 2004; Tintinger *et al*, 2005), is mobilized to the airways following inhalation of toxic gases and particles, and is the probable perpetrator of inflammation-related airway damage and dysfunction associated with inhalation of these agents (Bassett *et al*, 2000; Saldiva *et al*, 2002; Douwes *et al*, 2002). However, with a few exceptions (Zhang *et al*, 2001; Wang *et al*, 2003), relatively little is known about the potential of vanadium to alter the pro-inflammatory activities of these cells. In the current study, the effects of vanadium on the potentially harmful, prooxidative activities of human neutrophils

were investigated, with emphasis on the formation of hydroxyl radical, one of the most potent and toxic oxidants in biological systems (Cheng *et al*, 2002).

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Vanadium was used in the +2, +3, +4 and +5 valence states as vanadium (II) chloride, vanadium (III) chloride, vanadyl sulphate hydrate and sodium metavanadate with respective molecular weights of 121.85, 157.30, 163.00 and 121.93, all of which were purchased from Sigma-Aldrich (St Louis, MO, USA). According to the certificates of analysis provided by the manufacturer for the batches of each agent used in the current study the respective vanadium contents of each compound were 39.50, 31.40, 20.71 and 41.10% (titration with $KMnO_4$). These were dissolved in distilled water to give stock solutions of 10 mM for each agent, and used in the various assays described below at concentrations of 1-25 μM. Unless indicated, all other chemicals and reagents were also purchased from Sigma-Aldrich (St Louis, MO,USA).

2.2.2 Neutrophils

Purified neutrophils were prepared from heparinized (5 units of preservative-free heparin/ml) venous blood of healthy, non-smoking adult human volunteers and separated from mononuclear leukocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 g for 25 minutes at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 minutes. The neutrophils, which were routinely of high purity (>90%) and viability (>95%) were resuspended to $1x10^7$/ml in PBS and held on ice until used.

2.2.3 Superoxide anion production

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils ($1x10^6$/ml, final) were pre-incubated for 10 minutes in 800 μl indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$) containing 0.2 mM lucigenin. Following pre-incubation, 100 μl HBSS or vanadium (1.5-25 μM) in the +2, +3, +4 and +5 valence states followed immediately by addition of either 100 μl HBSS, or 100 μl of the synthetic chemoattractant N-formyl-L-leucyl-L-phenylalanine (FMLP, 1 μM final) or the phorbol ester, phorbol 12-myristate 13-acetate (PMA, 25 ng/ml), were added to control and experimental systems respectively and LECL responses recorded using an LKB Wallac 1251 chemiluminometer (Turku, Finland). LECL readings were integrated for 11 second intervals and recorded as mV x $seconds^{-1}$ ($mV.s^{-1}$).

The stock concentrations of FMLP and PMA were 0.5 mM and 1 mg/ml in dimethylsulphoxide (DMSO) and ethanol respectively, giving final concentrations of each solvent in the assays of reactive oxidant generation (superoxide anion, hydroxyl radical generation, MPO-mediated auto-iodination) of 0.02% (DMSO) and 0.0025% (ethanol).

2.2.4 Electron spin resonance (ESR) spectroscopy

A spin-trapping procedure was used to investigate the effects of vanadium in the +2, +3, +4 and +5 valence states on hydroxyl radical production using either a cell-free system with added hydrogen peroxide, or activated neutrophils. In the case of the cell-free system, the various vanadium compounds at a final concentration range of 1.5-25 μM were added to hydrogen peroxide (100 μM, final) in HBSS containing the hydroxyl radical spin trap, 1-pyroline N-oxide (DMPO, 20 mM final), in a final reaction volume of 1 ml, incubated at 37°C/5 minutes, and then analyzed for the DMPO-OH adduct using a Bruker EMX ESR analyzer (Bruker Biospin GmbH Rheinstetten, Germany), with the following parameter settings: receiver gain = 5.64 x 10^5; modulation amplitude = 2 Gauss; sweep width = 80 Gauss; sweep time = 41.94 ms; power = 25 mW. These experiments were performed in the presence and

absence of the hydroxyl radical scavenger, sodium benzoate (20 mM, final). The results are presented as either the spectra for individual experiments, or as the peak-to-peak intensities in arbitrary units.

In the case of cell-containing systems, neutrophils were preincubated for 10 min at 37°C in the presence and absence of the myeloperoxidase inhibitor, sodium azide at a final concentration of 760 μM (50 μg/ml), followed by addition in rapid succession of DMPO (20 mM, final), the vanadium compounds (1.5 - 25 μM) and either FMLP (1 μM), or an equivalent volume of HBSS to unstimulated control systems. When PMA (25 ng/ml) was used to activate the neutrophils, DMPO and vanadium were added to the cells 5 minutes after PMA. The tubes, which contained 2 x 10^6 neutrophils in a final volume of 2 ml HBSS, were incubated for either 2 minutes or 10 minutes at 37°C for FMLP- and PMA-activated systems respectively, followed by measurement of DMPO-OH. Superoxide dismutase (SOD, from bovine erythrocytes, 100 milliunits/ml final) was included in PMA-treated systems to protect the DMPO-OH adduct from superoxide during exposure of neutrophils to this potent activator of NADPH oxidase (Samuni *et al*, 1988).

To confirm the involvement of activated neutrophils in vanadium-mediated formation of hydroxyl radical, DMPO-OH adduct formation was measured using: i) a neutrophil-free system which contained all the other components of the assay, including SOD; and ii) a system in which varying concentrations of neutrophils (0.25, 1 and 4 x 10^6/ml, final) were activated with FMLP (1 μM) in the presence of vanadium (25 μM) in the +4 valence state.

In additional experiments, the effects of the NADPH oxidase and protein kinase C (PKC) inhibitors, diphenylene iodonium chloride (DPI, 10 μM final) and GF109203X (5 μM final) respectively, as well as those of the hydroxyl radical scavenger, sodium benzoate (20 mM, final), and catalase (from bovine liver, 500 units/ml, final) on hydroxyl radical formation by vanadium-treated neutrophils were also investigated.

2.2.5 Spectrofluorimetric detection of hydroxyl radical

As an alternative to ESR spectroscopy with DMPO, a spectrofluorimetric procedure based on the formation of catechol from salicyclate was used to detect hydroxyl radical formation in cell-free systems (Liu *et al*, 1997). The advantage of using this method is that it eliminates the complicating effects of superoxide on DMPO-OH, and can therefore be used to measure the possible involvement of superoxide in the conversion of vanadium from the +5 to the +4 valence states as described previously (Shi & Dalal, 1993; Zhang *et al*, 2001; Wang *et al*, 2003). In my experience, this method is suitable for detection of hydroxyl radical in cell-free systems, but not in cell-containing systems.

To investigate the potential of superoxide to reduce vanadium from the +5 to the +4 valence states, sodium metavanadate (100 μM) was added to an xanthine (0.9 mM, final) xanthine oxidase (66.6 mU/ml, final) superoxide/hydrogen peroxide-generating system with and without SOD (100 milliunits/ml, final) 5 minutes after initiation of the reaction and incubated for a further 15 minutes at room temperature in a final reaction volume of 2 ml HBSS containing 5 mM sodium salicyclate in the presence and absence of 100 μM NADPH (Liu *et al*, 1997). On completion of incubation, catechol was extracted from the incubation mixtures by addition of an equal volume of ethyl acetate. The ethyl acetate-extractable component was evaporated to dryness under a stream of nitrogen and reconstituted in 2 ml of borate buffer (0.3 M, pH 11.3) and 1 ml of a 1% aqueous solution of 2-cyanoacetamide as described previously (Liu *et al*, 1997). The tubes were then placed in a boiling water bath for 10 minutes, cooled immediately and fluorescence intensity recorded using a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 447 and 508 nm respectively (Liu *et al*, 1997). The effects of vanadium in the +2, +3 and +4 valence states on hydroxyl radical formation by the xanthine/xanthine oxidase system ± SOD was also investigated. Detection of hydroxyl radical using the xanthine/xanthine oxidase system required vanadium concentrations of 100 μM.

Positive control systems included vanadium (25 μM) in the +2, +3 and +4 valence states with added hydrogen peroxide (100 μM) in the absence and presence of sodium benzoate (20 mM), while systems consisting of xanthine/xanthine oxidase only, hydrogen peroxide only, sodium metavanadate + hydrogen peroxide, or the metals only served as negative controls.

2.2.6 Myeloperoxidase (MPO) activity

Auto-iodination of neutrophils was used to measure the activity of MPO following activation of neutrophils with FMLP (1 μM, final) in the absence and presence of vanadium, with the metal in its various valence states being used at a fixed, final concentration of 25 μM. Briefly, neutrophils (1 x 10^6) were preincubated for 10 minutes at 37°C in 0.8 ml HBSS containing 1 μCi of iodine-125 (as $Na^{125}I$, specific activity 17.4 Ci/mg, Perkin Elmer Life and Analytical Sciences, Boston, MA) and 25 nanomoles of cold carrier NaI. Following preincubation, 100 μl HBSS or vanadium (fixed, final concentration of 25 μM), followed immediately by addition of either 100 μl of HBSS (unstimulated systems), or 100 μl FMLP (1 μM, final) were added to each tube. The tubes, which contained a total volume of 1 ml, were incubated for 10 min at 37°C, after which the reactions were terminated and neutrophil proteins precipitated by addition of 3 ml 20% trichloracetic acid (TCA), after which they were pelleted by centrifugation and washed twice with TCA. The levels of radioactivity in the pellets were measured, and the results expressed as nmoles $^{125}I/10^7$ neutrophils.

The effects of the metals on the activity of purified MPO were investigated using a colorimetric procedure based on the oxidation of guaiacol. Reaction systems contained MPO (from human leukocytes, Sigma), guaiacol, and hydrogen peroxide at fixed final concentrations of 50 milliunits/ml, 4 mM and 5 mM respectively, with and without the metals, which were used at fixed final concentrations of 25 and 100 μM. The final volume in each well of a microtiter plate was 200 μl (all reactants in HBSS) and reactions were initiated by the addition of guaiacol/hydrogen peroxide, and MPO-mediated oxidation of guaiacol monitored at a wavelength of 450 nm in a microplate spectrophotometer.

2.2.7 Cellular ATP levels

To determine the effects of the test vanadium compounds (25 μM) on neutrophil viability, intracellular ATP concentrations were measured in cell lysates (1 x 10^6 cells/ml) following exposure to the metals for up to 6 hours at 37°C using a luciferin/luciferase procedure (Holmsen *et al*, 1972).

2.2.8 Expression and statistical analysis of results

With the exception of the results of the ESR spectroscopy experiments, some of which are shown as the spectra from individual experiments, the results of the other investigations are expressed as the mean values ± SEM. Statistical analysis of data was performed by using the Mann-Whitney U-test, and ANOVA where appropriate. A computer-based software system (Graph Pad Prism® 4, Graph Pad Software Inc. San Diego, CA) was used for analysis and *P* value of <0.05 was taken as significant. In the case of ESR results, relative peak intensity values were used to calculate levels of statistical significance.

2.3 Results

2.3.1 Hydroxyl radical generation in cell-free systems

The effects of vanadium in its various valence states in combination with hydrogen peroxide on hydroxyl radical formation using the ESR/DMPO system are shown in Figure 2.1 (page 56). Addition of hydrogen peroxide to vanadium in the +2, +3 and +4, but not the +5, valence states at all the concentrations tested (1.5-25 μM, the highest concentration shown in Figure 2.1 (page 56), resulted in an intense ESR spectrum consisting of the 1:2:2:1 quartet characteristic of the DMPO-OH adduct, compatible with hydroxyl radical formation. Inclusion of 20 mM benzoate markedly attenuated the formation of the DMPO-OH adduct. In the case of vanadium in the +2, +3 and +4 valence states, a clear dose response relationship was evident as can

be seen in Figure 2.2 (page 57), which depicts the ESR spectra generated following addition of vanadyl sulphate at concentrations of 1.5, 6.25 and 25 μM to 100 μM H_2O_2 in the presence of 20 mM DMPO, and in Table 2.1 (page 58), which shows the comparable data for the metal in the +2, +3 and +4 valence states, which indicate that the metal in the +3 valence state is most effective.

At the highest concentration of vanadyl sulphate (25 μM) shown in Figure 2.2 (page 57), it may seem that an additional spin adduct (s), possibly carbon-centred, is evident. Although the existence of such c-centred radicals is possible (Dikalov & Mason, 2001; Romero *et al*, 2003), their identity is not known. Moreover, the fact that the DMPO-OH radical signal increases to a greater degree than the peaks for these possible c-centred radicals, together with the benzoate data shown in Figure 2.1 (page 56), supports the primary involvement of the DMPO-OH radical.

Using the spectrofluorimetric procedure for the detection of hydroxyl radical, addition of vanadium in the +2, +3 and +4 valence states to hydrogen peroxide in the presence of sodium salicyclate resulted in the formation of catechol, compatible with the generation of salicylate-reactive hydroxyl radical, as shown in Figure 2.3 (page 59). Again, vanadium in the +5 valence state was unreactive. This procedure was also used to probe the possible involvement of superoxide in the conversion of vanadium from the +5 to the +4 valence states, in preference to the ESR/DMPO procedure because of the sensitivity of the DMPO-OH adduct to superoxide (Samuni *et al*, 1988). These results, which are shown in Figure 2.4 (page 60), demonstrate formation of hydroxyl radical on exposure of vanadium in the +2, +3 and +4 valence states to the xanthine/xanthine oxidase superoxide/hydrogen peroxide-generating system. However, formation of hydroxyl radical was not detected in the system containing vanadium in the +5 valence state, either in the absence or the presence of NADPH.

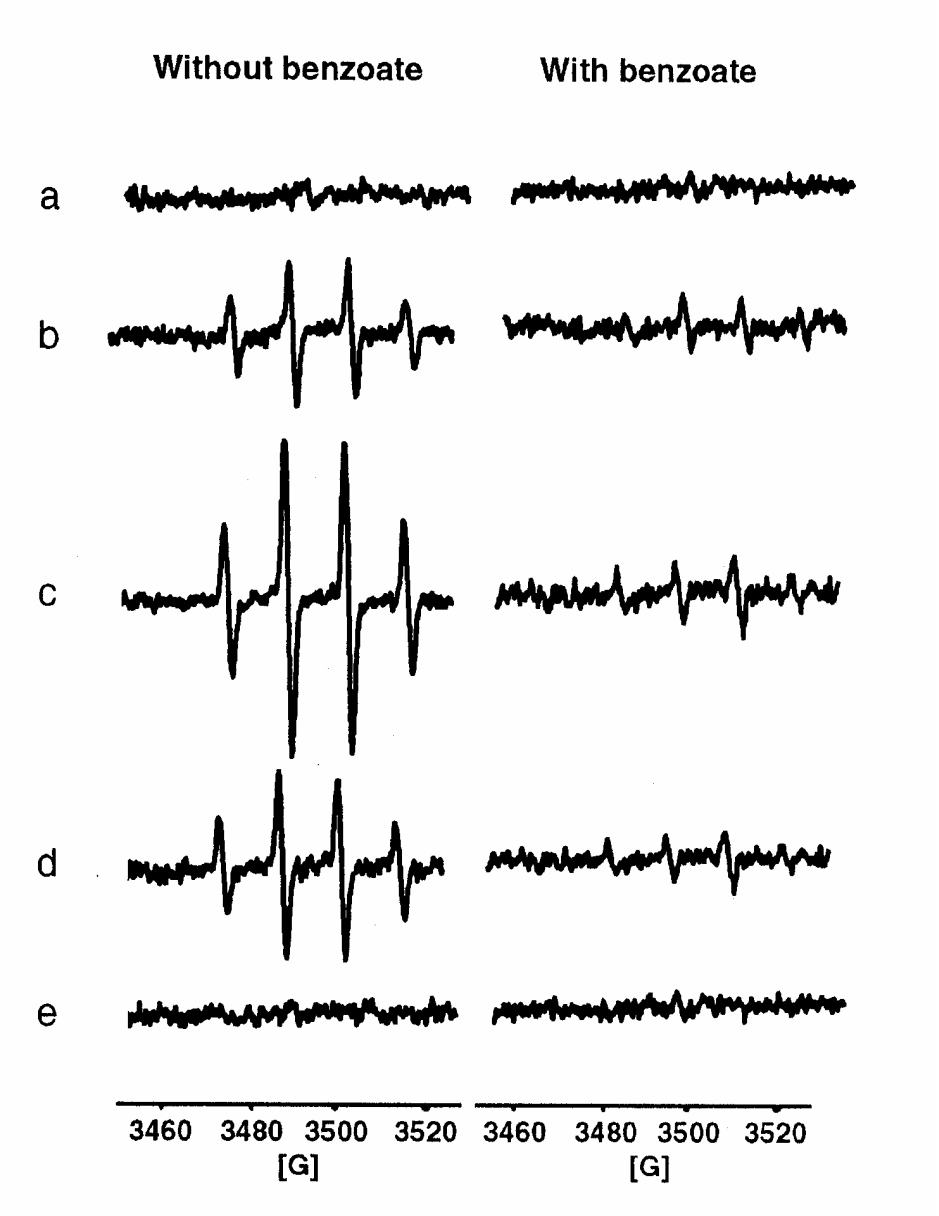

Figure 2.1

ESR spectra following the addition of 100 μM hydrogen peroxide to 20 mM DMPO in HBSS only (a) and DMPO + 25 μM vanadium in the +2 (b), +3 (c), +4 (d), and +5 (e) oxidation states in the absence or presence of 20 mM sodium benzoate. Spectra are representative of four separate experiments.

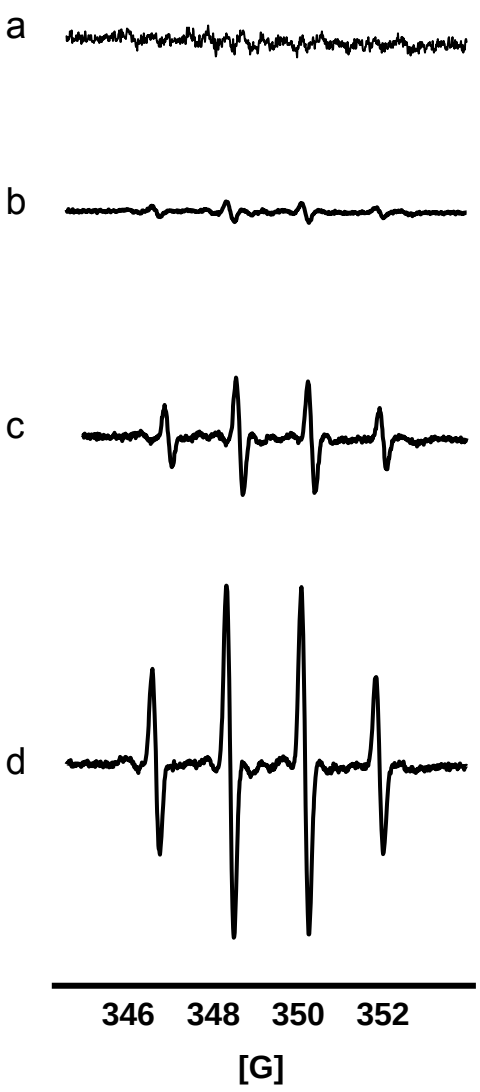

Figure 2.2

ESR spectra following the addition of 100 μM hydrogen peroxide to 20 mM DMPO in HBSS only (a) and DMPO + vanadium in the +4 valence state at concentrations of 1.5 μM (b), 6.25 μM (c), and 25 μM (d). Spectra are representative of two separate experiments.

Table 2.1

Comparison of the peak intensities of the DMPO-OH spectra generated following the addition of varying concentrations of vanadium (1.5, 6.25, 25 μM) in the +2, +3, and +4 valence states to DMPO (20 mM) and hydrogen peroxide (100 μM).

	Peak intensities observed		
	1.5 μM	**6.25 μM**	**25 μM**
V^{2+}	19,214 ± 1293	41,460 ± 627	102,058 ± 5134
V^{3+}	29,302 ± 6173	60,717 ± 888	161,417 ± 7506
V^{4+}	19,031 ± 4555	42,097 ± 2966	121,583 ± 3126

Results of three separate experiments presented as the mean peak-to-peak intensity values in arbitrary units ± SE. The mean background value for the metal-free, control system (hydrogen peroxide + DMSO) was 8024 ± 488.

Importantly, inclusion of SOD did not affect the conversion of salicylate to catechol by xanthine/xanthine oxidase in the presence of vanadium in the +2, +3 and +4 valence states. The values for systems containing xanthine/xanthine oxidase without vanadium (100 μM) and in the presence of the metal in the +2, +3, +4 and +5 valence states in the absence of SOD were 11 ± 0.5, 121 ± 8.5, 191 ± 3, 98 ± 3.5, and 11.5 ± 0.5 fluorescence intensity units respectively; the corresponding values in the presence of SOD were 13 ± 1, 120 ± 12.5, 185 ± 0.5, 124 ± 3.5 and 11.5 ± 0.5 respectively (data from 2 determinations ± SEM). These results confirm that vanadium (+2, +3, and +4) interacts with hydrogen peroxide, not superoxide, to generate hydroxyl radical. These results are shown in Figures 2.3 and 2.4 (page 59 and 60, respectively).

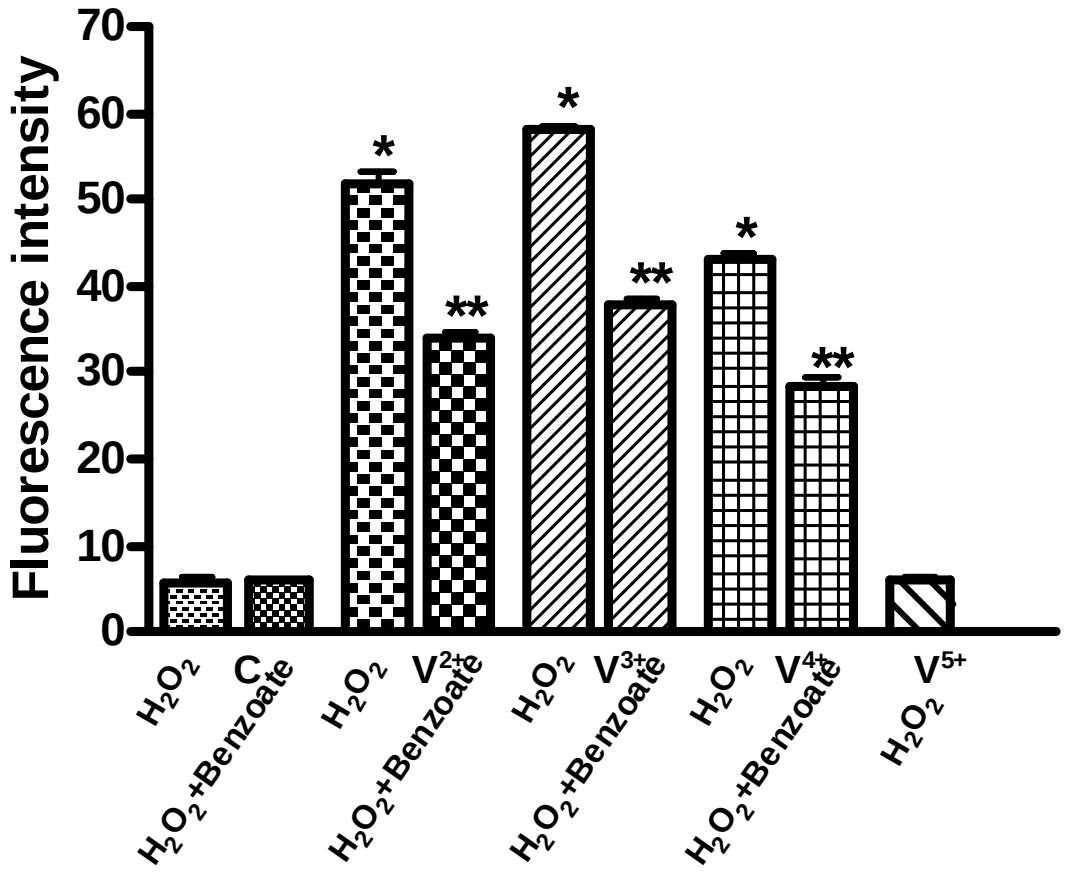

Figure 2.3

The effects of addition of 100 μM hydrogen peroxide to vanadium in the +2, +3, +4, and +5 valence states (all at 25 μM) in the absence and presence of 20 mM sodium benzoate on formation of hydroxyl radical, using the spectrofluorimetric procedure. The results are expressed as the mean values of six different experiments ± SE; ***p*<0.05 for comparison of the vanadium-treated systems with the vanadium-free systems and ****p*<0.05 for comparison of matched systems without and with sodium benzoate.

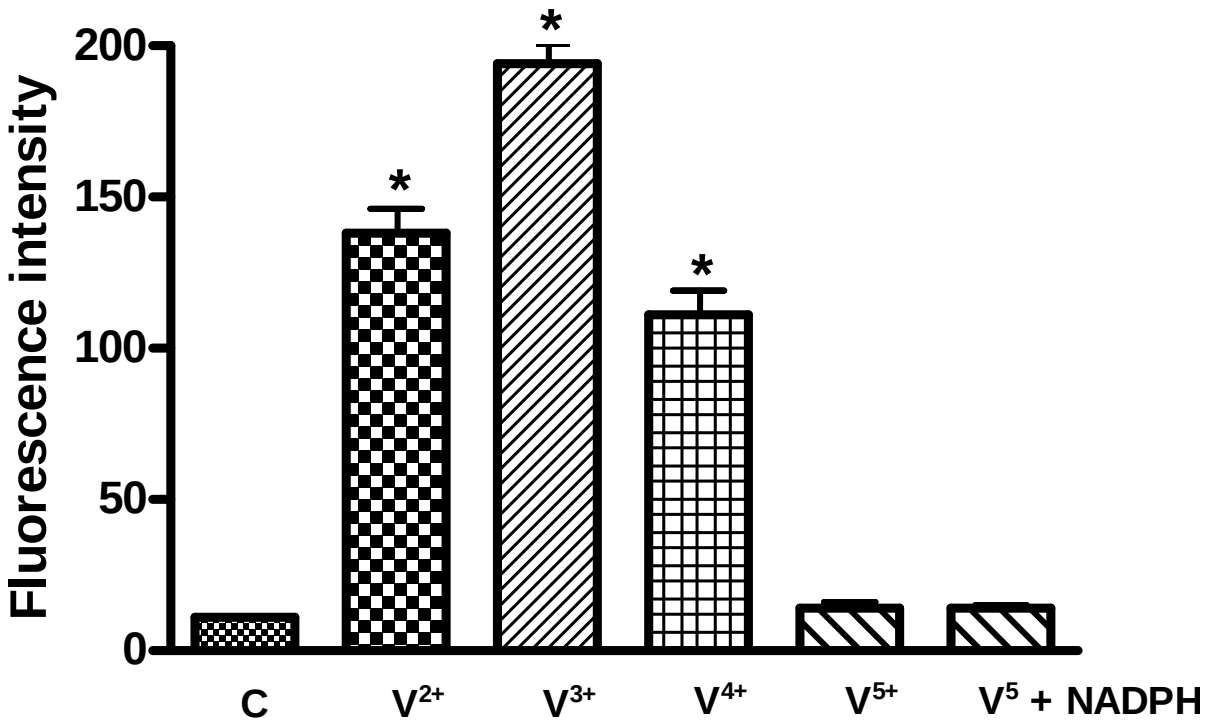

Figure 2. 4

Effects of addition of vanadium in the +2, +3, +4, and +5 valence states (all at 100 μM) to a xanthine/xanthine oxidase superoxide/hydrogen peroxide-generating system on formation of hydroxyl radical, using the spectrofluorimetric procedure. The effects of vanadium in the +5 valence state were measured in the absence and presence of 100 μM NADPH. The results are expressed as the mean values of three to six different experiments ± SE; ***p* <0.05 for comparison with the vanadium-free control system.

2.3.2 Superoxide production by activated neutrophils

These results are shown in Table 2.2. Vanadium in the four different valence states tested, and at all concentrations used (1.5-25 μM, only data for the highest concentration shown), did not affect the lucigenin-enhanced chemiluminescence (LECL) responses of neutrophils activated with either FMLP or PMA.

No significant responses above basal level (lucigenin only) were observed in cell-free systems following addition of H_2O_2 (100 μM) alone, or H_2O_2 + vanadium (25 μM for each of the 4 valence states) to lucigenin, compatible with lack of reactivity of hydroxyl radical with lucigenin (Yildiz & Demiryurek, 1998; Myhre *et al*, 2003).

Table 2.2

Effects of vanadium (in the four oxidation states) on FMLP- and PMA-activated superoxide production by neutrophils.

System	PMA-activated LECL (mV/s)	FMLP-activated LECL (mV/s)
Control	2513 ± 134	1143 ± 157
V^{2+}	2491 ± 136	1057 ± 48
V^{3+}	2565 ± 142	1249 ± 222
V^{4+}	2477 ± 137	1104 ± 50
V^{5+}	2585 ± 125	1161 ± 89

Results are expressed as the mean peak values ± SE of 3 – 11 experiments measured at around 40 seconds and 4 minutes for FMLP- and PMA-activated neutrophils, respectively. The corresponding values for unstimulated cells were 287 ± 33 and 230 ± 4 mV/s, respectively.

2.3.3 Hydroxyl radical production by activated neutrophils

The effects of the various vanadium compounds on the generation of hydroxyl radical by neutrophils activated with FMLP in the presence and absence of the MPO inhibitor, sodium azide, are shown in Figure 2.5 (page 63). Hydroxyl radical formation was detected following exposure of neutrophils to FMLP, which was of similar magnitude in both the absence and presence of sodium azide. Inclusion of vanadium in the +2, +3 and +4 valence states significantly ($p < .05$) increased the formation of hydroxyl radical by FMLP-activated neutrophils, with additional and significant ($p < .05$) augmentation observed in the presence of sodium azide (Figure 2.5, page 63). However, vanadium in the +5 valence state did not affect the magnitude of hydroxyl radical formation by neutrophils in either the absence or the presence of sodium azide (Figure 2.5, page 63).

Importantly, the magnitudes of hydroxyl radical formation by FMLP-activated neutrophils treated with 25 μM vanadium in the +4 oxidation state (in the absence of azide) shown in Figure 2.5 (page 63), were considerably attenuated and augmented when the neutrophil concentration of 1 x 10^6/ml was decreased or increased to 0.25 and 4 x 10^6/ml respectively. The corresponding mean percentages of the control system (with 1 x 10^6/ml neutrophils) being 6% and 353% respectively.

The effects of vanadium in all four valence states on the formation of hydroxyl radical by neutrophils activated with PMA are shown in Figure 2.6 (page 64) (all systems shown contained 100 mU/ml SOD because no spectra were detected in the absence of the enzyme). Slight formation of hydroxyl radical was detected in the vanadium-free, PMA-activated control systems, in both the absence and the presence of sodium azide. Addition of vanadium in the +2, +3 and +4, but not the +5, valence states at all concentrations tested (1.5 - 25 μM, only data for the highest concentration shown) was accompanied by significantly ($p <.05$) increased DMPO-OH signals, with additional and significant ($p <.05$) enhancement in the presence of sodium azide.

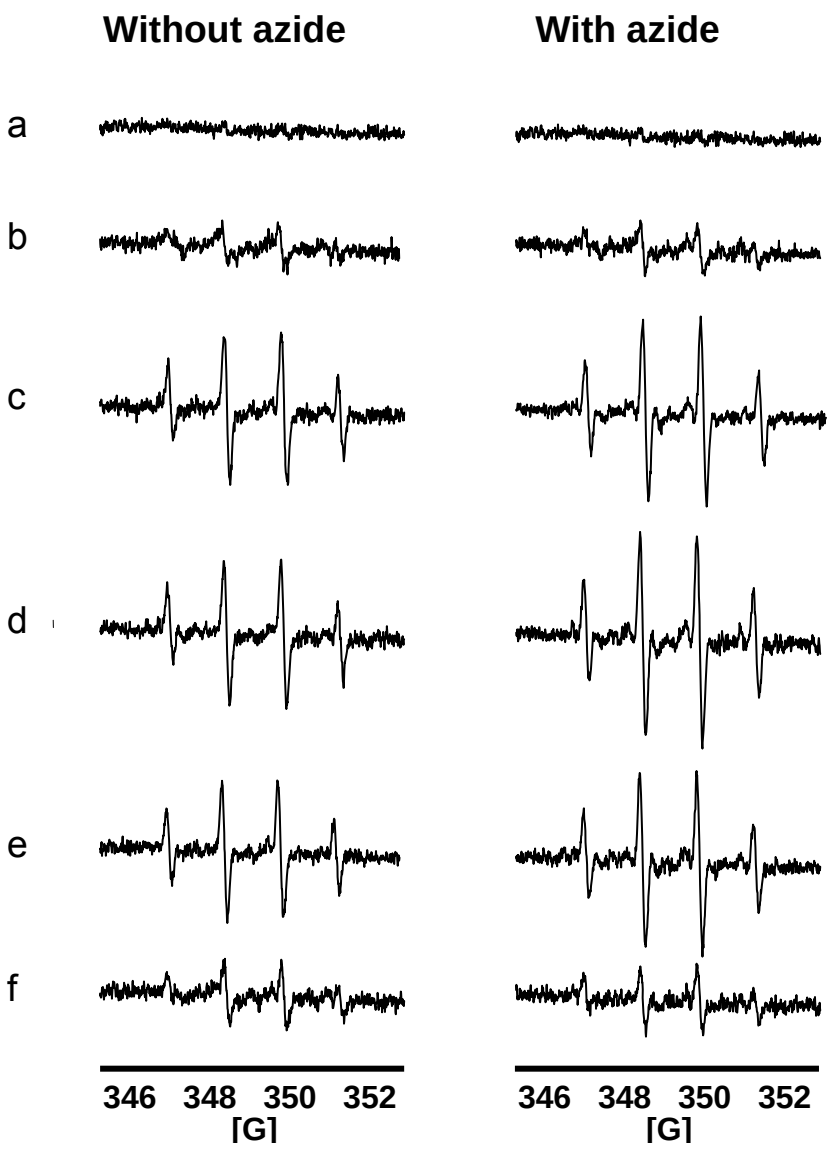

Figure 2.5

ESR spectra of unstimulated neutrophils + 20 mM DMPO (a) and those of FMLP (1 μM)-activated neutrophils in the absence of vanadium (b) and in the presence of vanadium in the +2 (c), +3 (d), +4 (e), and +5 (f) valence states in the absence and presence of sodium azide (760 μM). Spectra are representative of three separate experiments, each with triplicate determinations for each system.

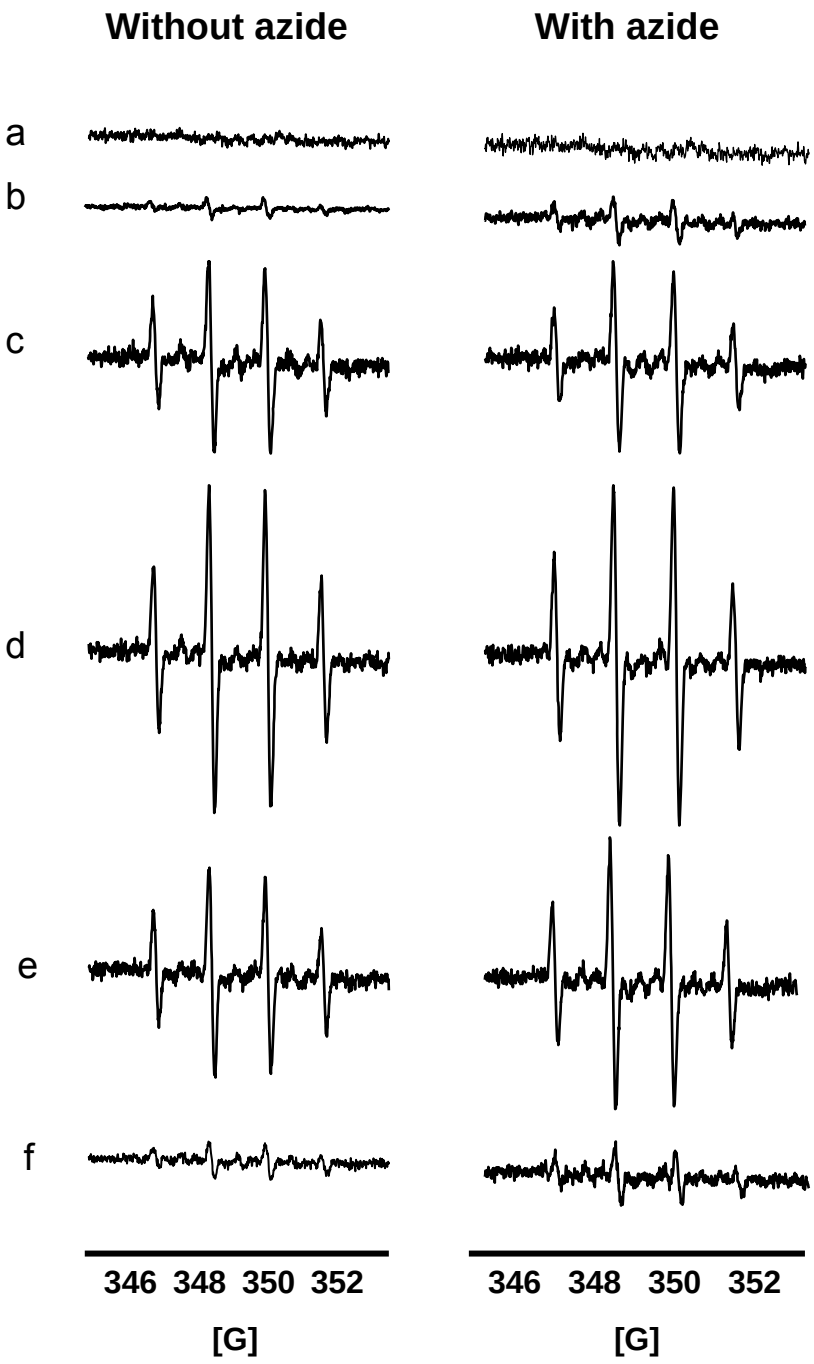

Figure 2.6

ESR spectra of unstimulated neutrophils + 20 mM DMPO (a) and those of PMA (25 ng/ml)-activated neutrophils in the absence of vanadium (b) and in the presence of vanadium in the +2 (c), +3 (d), +4 (e), and +5 (f) valence states in the absence and presence of sodium azide (760 μM). Spectra are representative of three separate experiments, each with triplicate determinations for each system.

As shown in Figure 2.7 (page 66), sodium benzoate partially neutralized hydroxyl radical generation by neutrophils activated with PMA in the presence of vanadium in the +4 valence state (with SOD, without sodium azide), while DPI (inhibitor of NADPH oxidase), GF109203X (inhibitor of protein kinase C) and catalase completely attenuated formation of the DMPO-OH adduct with no spectra observed.

In cell-free control systems containing all components of the assay system (except neutrophils) formation of the DMPO-OH adduct was not detected.

2.3.4 Activity of myeloperoxidase

The effects of the various vanadium compounds (all at 25 μM) on MPO-mediated auto-iodination of FMLP-activated neutrophils, as well as on the oxidation of guaiacol by purified MPO (compounds at 25 and 100 μM) are shown in Figures 2.8 and 2.9 (pages 67 and 68, respectively). Inclusion of 25 μM vanadium in the +2, +3 and +4 valence states, but not the +5 valence state, significantly (p <.05) decreased FMLP-mediated auto-iodination of neutrophils, as well as the oxidation of guaiacol by purified MPO.

2.3.5 Cellular ATP levels

Exposure of neutrophils to the various vanadium compounds (25 μM) for up to 6 hours at 37°C did not affect cellular ATP levels, demonstrating lack of cytotoxicity of the metal at the concentrations used in the various experiments described in the current study. The values for control cells and those exposed to vanadium in the +2, +3, +4 and +5 valence states for 6 hours at 37°C were 26.5 ± 5.5, 25.3 ± 5.3, 26.4 ± 5.3, 28.5 ± 2.8 and 22.9 ± 3.2 nmoles ATP/10^7 cells respectively.

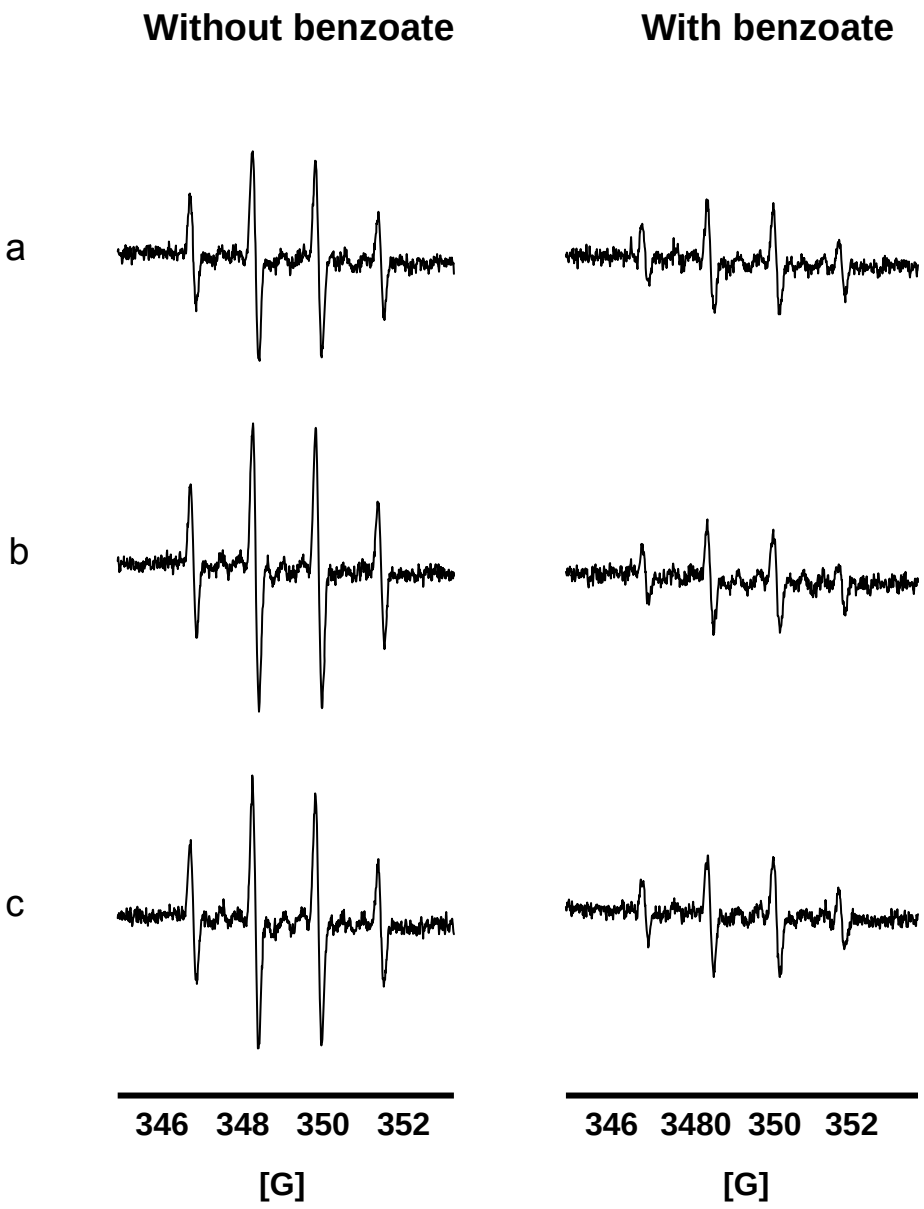

Figure 2.7

Results from three experiments showing the paired ESR spectra of PMA (25 ng/ml)-activated neutrophils + 20 mM DMPO, treated with 25 μM vanadium in the +4 valence state in the absence (left) and presence (right) of 20 mM sodium benzoate.

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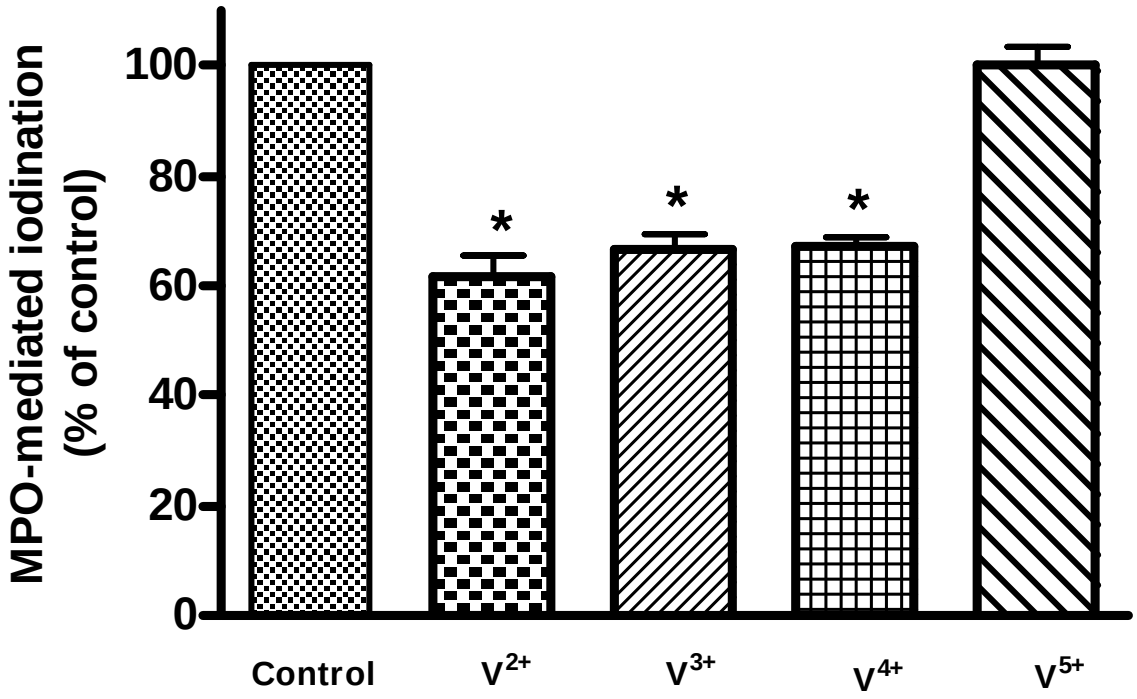

Figure 2.8

Effects of vanadium in the +2, +3, +4, and +5 valence states (25μM) on FMLP-activated MPO-mediated autoiodination of intact neutrophils. The results of three to six experiments with four to five replicates for each system are presented as the mean percentage of the corresponding metal-free control system ± SEM.

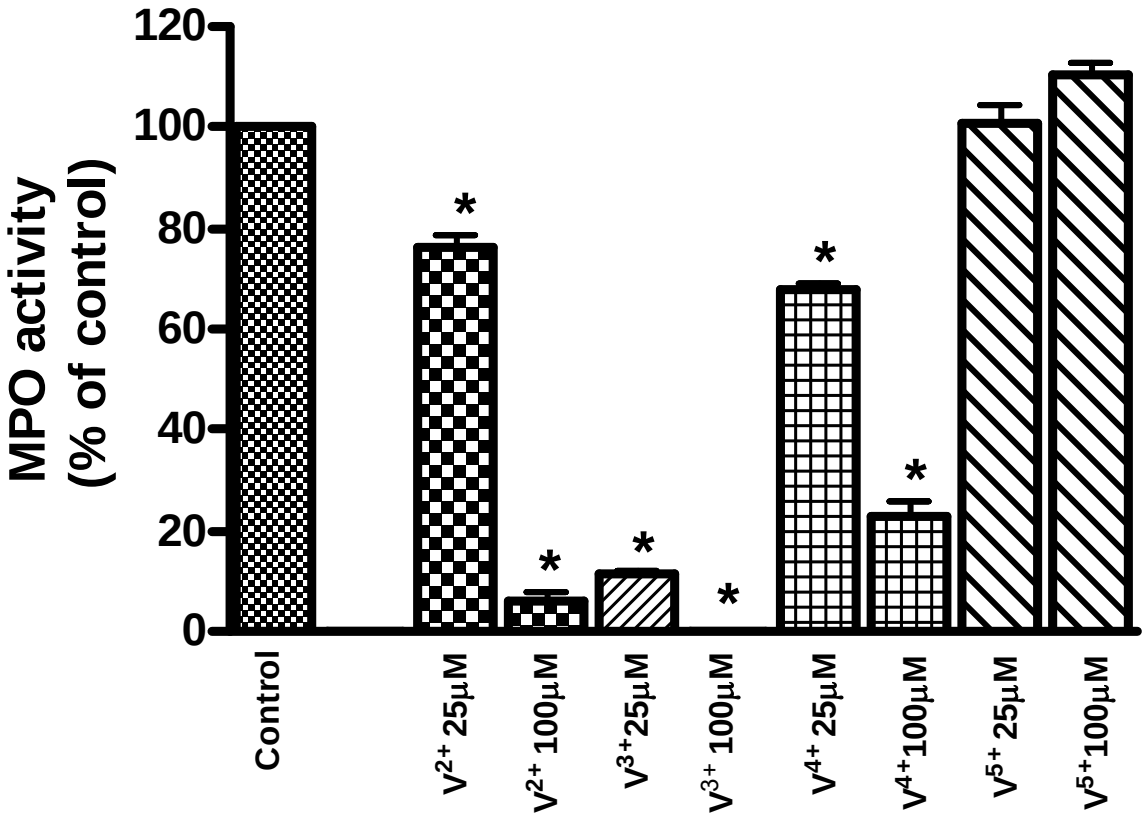

Figure 2.9

Effects of vanadium in the +2, +3, +4, and +5 valence states (25 μM) on the oxidation of guaiacol by a cell-free system containing purified MPO and H_2O_2 (metal compounds at 25 and 100 μM). The results of three to six experiments with four to five replicates for each system are presented as the mean percentage of the corresponding metal-free control systems ± SEM. * $p < 0.05$

2.4 Discussion

The pathophysiological relevance of hydroxyl radical production by human neutrophils has been questioned, largely because of regulatory mechanisms which restrict the availability of heavy metals, particularly iron, required to participate in the Haber-Weiss and/or Fenton reactions, as well as the efficiency of MPO in removing hydrogen peroxide (Samuni *et al*, 1988; Cohen *et al*, 1988; Britigan *et al*, 1989; Britigan *et al*, 1990). Nevertheless, situations may arise in which regulatory mechanisms are subverted, resulting in significant formation of hydroxyl radical by activated neutrophils (Winterbourn, 1986; Miller & Britigan, 1995; Andersen *et al*, 2003). The results of the current study demonstrate that exposure of neutrophils to vanadium favours hydroxyl radical formation by these cells.

Activation of neutrophils with the chemoattractant, FMLP, was accompanied by detectable, albeit modest formation of hydroxyl radical. Inclusion of vanadium in the +2, +3 and +4, but not the +5 valence states, resulted in significant augmentation of hydroxyl radical formation by FMLP-activated neutrophils. Detection of vanadium-mediated catalysis of hydroxyl radical formation by FMLP-activated neutrophils did not require inclusion of superoxide dismutase, while addition of the MPO inhibitor, sodium azide, caused a further increment in hydroxyl radical formation in vanadium (+2, +3, or +4)-treated systems. Apart from underscoring the prooxidative interactions of vanadium (+2, +3 and +4) with human neutrophils, these observations demonstrate that the levels of superoxide generated during the relatively brief activation of NADPH oxidase following exposure of the cells to FMLP are insufficient to inactivate the DMPO-OH spin adduct, while the metal appears to compete efficiently with MPO for hydrogen peroxide. The augmentative effects of inclusion of sodium azide, although modest, were nevertheless statistically significant, and are probably due to increased availability of hydrogen peroxide to interact with vanadium in a Fenton-type reaction. Because MPO negatively modulates superoxide generation by activated phagocytes (Locksley *et al*, 1983), increased generation of hydrogen peroxide by these cells may also contribute to the augmentative reaction of sodium azide.

In the case of vanadium-free control cells exposed to PMA, an extremely potent activator of NADPH oxidase, low-level formation of hydroxyl radical was

detected, which was dependent of the inclusion of superoxide dismutase. However, treatment of PMA-activated cells with vanadium in the +2, +3 and +4, but not the +5, valence states resulted in substantial formation of hydroxyl radical, detection of which was strictly dependent on inclusion of superoxide dismutase and was potentiated by sodium azide. Importantly, the stimulatory effects of vanadium (+2, +3, +4) on hydroxyl radical formation by activated neutrophils were attenuated by diphenylene iodonium chloride, GF 109203X and sodium benzoate, inhibitors of NADPH oxidase and protein kinase C, and a scavenger of hydroxyl radical respectively, as well as by catalase. The complete attenuation of hydroxyl radical formation observed when vanadium (+4)-treated, activated neutrophils were exposed to catalase, indicates that formation of hydroxyl radical is an exclusively extracellular event.

Using both ESR and spectrofluorimetric procedures, similar effects of vanadium on hydroxyl radical formation were also observed using a cell-free system consisting of the metal and added hydrogen peroxide. In agreement with previous reports (Keller *et al*, 1989; Carmichael, 1990), these observations are compatible with a Fenton-type mechanism, whereby vanadium (in the +2, +3 and +4 valence states) reduces hydrogen peroxide with consequent formation of hydroxyl radical. Using the spectrofluorimetric system, which is not prone to interference by superoxide, unlike the ESR/DMPO system, hydroxyl radical formation was also detected following the addition of vanadium (+2, +3, +4) to a xanthine/xanthine oxidase superoxide- and hydrogen peroxide-generating system. However, vanadium in the +5 valence state was completely unreactive, even in the presence of added NADPH. Taken together with the results of experiments using FMLP-activated neutrophils (without added superoxide dismutase), these results suggest that over the relatively short time course of these experiments, and at the concentrations of vanadium, cells and enzyme/substrate used, that superoxide, in both the presence and absence of NADPH, does not convert the metal from the +5 to the hydrogen peroxide-reactive +4 valence state.

While in agreement with several previous reports (Liochev *et al*, 1989a; Liochev *et al*, 1989b; Liochev *et al*, 1991), these observations that vanadium in the +5 valence state is not converted by superoxide-dependent mechanisms to the +4 valence state are at variance with other reports in which an alveolar cell line (Zhang

et al, 2001), and murine bronchoalveolar lavage cells, consisting predominantly of macrophages (Wang *et al*, 2003), or a cell-free system consisting of xanthine/xanthine oxidase + NADPH (Shi & Dalal, 1993) were used. These differences may reflect the different cell types used in the various studies, as well as the concentrations of vanadium (+5) which were higher than those used in the current study (Shi & Dalal, 1993; Zhang *et al*, 2001; Wang *et al*, 2003). The exact mechanisms by which vanadium is transformed from the +5 to the +4 valence states *in vivo* remain to be established, but may involve reduction by glutathione and/or NADPH-dependent flavoenzymes intracellularly (Barceloux, 1991b; Shi & Dalal, 1991).

Although vanadium in the +5 valence state did not promote the formation of hydroxyl radical by activated neutrophils, reagent hydrogen peroxide, or an enzymatic superoxide/hydrogen peroxide system, several alternative mechanisms exist by which vanadate may function as an occupational and environmental toxin. These are i) by conversion to the +4 valence state *in vivo*, favouring formation of hydroxyl radical; ii) via inhibition of plasma membrane ATPases, particularly Na^+, K^+-ATPase (Sabbioni *et al*, 1991), iii) by activating transcription factors and synthesis of pro-inflammatory cytokines (Chen *et al*, 1999; Chong *et al*, 2000; Huang *et al*, 2001; Nadadur *et al*, 2002), and iv) by inhibition of tyrosine phosphatases (Grinstein *et al*, 1990; Trudel *et al*, 1991; Zhao *et al*, 1996; Samet *et al*, 1997), which in the case of phagocytic cells, results in prolonged activation of NADPH oxidase. With respect to neutrophils, this latter effect requires permeabilization of the cells (Grinstein *et al*, 1990; Trudel *et al*, 1991) which explains why it was not observed in the current study.

Interestingly, vanadium in the +2, +3 and +4 valence states, but not the +5 valence state, decreased the activity of MPO both in intact neutrophils and in a cell-free system containing purified enzyme. Although the mechanisms by which vanadium in these oxidation states decreases the activity of MPO have not been identified, competition between the metal and the enzyme for hydrogen peroxide appears to be the most probable mechanism. Effective competition of the metal with MPO may also explain why hydroxyl radical formation by FMLP-activated neutrophils was detected in the absence of the MPO inhibitor, sodium azide.

The concentrations of vanadium used in the current study, which ranged from 0.2 - 4 μg/ml (1.5 - 25 μM), are comparable with those encountered in both the environmental and occupational settings. In 1973, the median level of vanadium in ambient air was reported to be 0.62 $\mu g/m^3$ in American cities with a high level of consumption of fossil fuels, while in localities in close proximity to metallurgical industries, or industries producing vanadium metal or compounds, atmospheric concentrations of the metal were reported to be around 1 $\mu g/m^3$ and several $\mu g/m^3$ respectively (reviewed in Barceloux, 1999b). Boiler cleaners are exposed to extremely high levels of vanadium oxides, which may reach concentrations of up to 500 mg/m^3 in dusts (Barceloux, 1999b).

Hydroxyl radical is one of the most damaging and reactive free radicals generated in biological systems (Cheng *et al*, 2002). However, its formation by phagocytes via Haber-Weiss/Fenton mechanisms *in vivo* is stringently controlled, primarily by binding proteins which limit the availability of heavy metals, especially copper and iron, as well as removal of hydrogen peroxide by MPO. The results of the current study have demonstrated a mechanism whereby exposure to vanadium enables these anti-oxidative mechanisms to be subverted, favouring hydroxyl radical formation by activated neutrophils. This is achieved by a Fenton reaction assisted by effective competition of the metal with MPO for hydrogen peroxide. Although the relevance of these findings to the pathogenesis of respiratory symptoms and dysfunction which may accompany environmental and occupational exposure to vanadium remain to be established, they do suggest that individuals with preexisting airways inflammation such as cigarette smokers, asthmatics, and those with chronic obstructive pulmonary disorders may be at highest risk for vanadium toxicity. This might also be the case in cardiovascular disease, as formation of the artherosclerotic plaques is augmented if LDL is oxidised. Only oxidised LDL can be taken up by macrophages, subsequently leading tp the formation of the plaque (Steinberg *et al*, 1989). Any chronic inflammatory condition exposes the individual to increased eactive oxidant species, including hydrogen peroxide, which through the catalytical properties of vanadium, can than be converted to the highly toxic hydroxyl radical.