Chapter 3

Activation of Nuclear Factor-Kappa B (NF-κB) in Human Neutrophils

Hypothesis

Cobalt, palladium, platinum, and vanadium initiate the signalling pathway leading to the activation of cytosolic kinases and other transcription factors responsible for the subsequent phosphorylation and degradation of IκB, the nuclear translocation of NF-κB, and the transcription of inflammatory genes introducing the production of IL-8.

Aim

The aim was to investigate the effects of the metals on the phosphorylation of cytosolic proteins, the nuclear translocation of NF-κB, and the production of IL-8 in human neutrophils, using a Bio-Plex suspension array system and an electrophoretic mobility shift assay (EMSA).

3.1 Introduction

NF-κB, in unison with several other transcription factors, plays an important role in inflammatory processes, which can be beneficial as well as detrimental to the host. Controlled activation of NF-κB is necessary for effective host defences, while increased or prolonged activation contributes to tissue damage in various pathological conditions. In its inactivated form, NF-κB, coupled to the inhibitory protein IκB, is present in the cytosol from where it translocates into the nucleus, following stimulation of the cell, with subsequent phosphorylation and degradation of IκB (Karin & Ben-Neriah, 2000). Different signalling pathways have been identified which vary according to the type of cell, as well as the stimulus and its corresponding receptor (Deva *et al*, 2003; Jiang *et al*, 2003; Sandor *et al*, 2003; Santoro *et al*, 2003; Asehnoune *et al*, 2004). After co-operative binding of several transcription factors, gene transcription is induced. In 1999 more than 150 different stimuli were already recognized which induced NF-κB activation, while active NF-κB was found to promote the expression of as many target genes (Pahl, 1999).

Activation of NF-κB follows not only exposure to bacteria and viruses and their proteins, but also occurs in response to different stress situations. The active NF-κB

transcription factor promotes the expression of many target genes, leading to the transcription of proteins participating in host immune and inflammatory responses (Holloway *et al,* 2001). These proteins include different cytokines and chemokines, as well as receptors required for immune recognition, such as MHC molecules, proteins involved in antigen presentation and receptors required for neutrophil adhesion and transmigration across the blood vessel wall (Pahl, 1999). Many viruses have NF-κB binding sites in their viral promoters, and low levels of NF-κB activation probably contribute to the mechanism by which some viruses, such as EBV, HSV, CMV and HIV-1, sustain chronic infection (Santoro *et al*, 2003).

Many activators of NF-κB have been described which are not bacterial or viral pathogens, and NF-κB can generally be understood to be a regulator of stress responses. NF-κB activity is induced during various pathophysiological stress conditions such as ischemia/reperfusion, liver regeneration and hemorrhagic shock. Physical stress in the form of irradiation, as well as oxidative stress to cells also induce NF-κB and, a large variety of stress response genes are in turn activated by NF-κB (Pahl, 1999).

NF-κB is also activated by both environmental stresses, such as heavy metals or cigarette smoke, and by therapeutic drugs (Pahl, 1999). Several studies have investigated the effects of metals on the activation and translocation of NF-κB in different cell lines. Goebeler *et al* (1995) found a strong increase in NF-κB DNA binding after exposing human umbilical vein epithelial cells (HUVEC) to nickel and cobalt chloride, which was confirmed by Sultana *et al* (1999) who also found increased expression of VCAM-1 and activation of MAP kinase. When alveolar epithelial (A549) cells were exposed to particulate air pollution matter/diameter 10 microns (PM_{10}) consisting of ultrafine particles in association with transition metals and endotoxins, increased activation of NF-κB was found (Jiménez *et al*, 2000). In macrophages, cobalt, as well as chromium ions, induced TNF-α secretion and cytotoxicity, while in synoviocytes DNA binding activity of NF-κB, as well as cytokine production was upregulated (Catelas *et al,* 2003; Niki *et al*, 2003). Exposure of monocytes to palladium caused a significant increase in activated NF-κB (Lewis *et al*, 2003); in another study, platinum salts were found to enhance cytokine release, while

palladium salts inhibited the release of IFN-γ, -TNF-α and -IL-5 by PHA-activated peripheral blood mononuclear cells (PBMC) (Boscolo *et al*, 2004) in contrast to the study by Lewis *et al* (2003).

Although pro-oxidative interactions between heavy metals and neutrophils have been described, little is known about the effect which heavy metals may have on the degradation of IκB and activation of NF-κB in human neutrophils. For example, cobalt was reported to increase the ROS generating capacity of neutrophils, as well as the serum opsonic activity (Ono *et al*, 1994). More recently, platinum and palladium were found to enhance the reactivity of superoxide anion generated by human neutrophils *in vitro* (Theron *et al*, 2004), while cobalt was found to potentiate the reactivity of neutrophil-derived H_2O_2 (Ramafi *et al*, 2004). As described in chapter 2 of this thesis, vanadium promotes hydroxyl radical formation by activated neutrophils. As mentioned in the literature review, oxidative stress plays a general role in the induction of signalling pathways leading to phosphorylation and degradation of IκB. In the case of the neutrophil, the involvement of ROS in facilitating nuclear translocation of NF-κB and subsequent production of the proinflammatory cytokines TNF-α, MIP-2, and IL-1β was described following activation of Toll-like receptor 4 (Asehnoune *et al*, 2004).

The research described in this chapter consists of two distinct, but related phases. The first of these was focussed on developing an electrophoretic mobility shift assay (EMSA) for detection of nuclear translocation of NF-κB proteins in neutrophils, which necessitated identification of a reliable, positive control system. This was achieved by using the pneumococcal, pore-forming cytotoxin, pneumolysin, which causes a sustained influx of extracellular Ca^{2+} into these cells which is associated with, and appears to be a prerequisite for toxin-activated synthesis of IL-8 (Cockeran *et al,* 2001a; Cockeran *et al*, 2002b). The relationship between sustained increases in cytosolic Ca^{2+}, activation of NF-κB and synthesis of IL-8 is well-recognized (Kuhns & Gallin, 1995; Kuhns *et al*, 1998; Dolmetsch *et al*, 1997). The involvement of extracellular Ca^{2+} in pneumolysin-mediated activation of NF-κB in neutrophils was probed by addition of EGTA, a chelator of extracellular Ca^{2+}, to the cells, and by pretreatment of the cells with the omega-3 polyunsaturated fatty acid

docosahexaenoic acid (DHA). The latter agent antagonizes influx of Ca^{2+} via interference with various types of Ca^{2+} channels (Wang *et al,* 2003; Honen *et al,* 2003) and DHA-mediated inhibition of NF-κB has been observed in LPS-activated macrophages (Komatsu *et al*, 2003; Lee *et al,* 2003; Park *et al*, 2004).

Having developed the NF-κB assay for neutrophils, the objectives of phase 2 were to investigate the effects of the metals on: i) activation and translocation of NF-κB in neutrophils, as well as production of IL-8; and ii) cytosolic signalling cascades which converge on NF-κB (ERK1&2, EGFR, IκB, JNK, p38MAPK), as well as on activation of other transcription factors which cooperate with NF-κB to optimize gene transcription (STAT3, ATF-2).

3.2 Materials and methods

3.2.1 Chemicals and reagents

Cobalt(II) chloride hexahydrate (Co^{2}), platinic chloride [hydrogen hexachloroplatinate (IV) (Pt^{4+}), palladium (II) chloride (Pd^{2+}), vanadium (II) chloride (V^{2+}), vanadium (III) chloride (V^{3+}), vanadyl sulphate hydrate (V^{4+}) and sodium metavanadate (V+5) were purchased from Sigma-Aldrich (St Louis, MO, USA). These were dissolved in distilled water to give stock concentrations of 10mM and used in the assays described below at concentrations of 25 μM for each metal. Unless otherwise indicated all other chemicals and reagents were also obtained from Sigma-Aldrich (St Louis, MO, USA). Recombinant IL-8 was also purchased from Sigma-Aldrich, while recombinant pneumolysin, prepared as described previously (Saunders *et al*, 1989), was kindly provided by Prof. T.J. Mitchell, Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, UK.

3.2.2 Neutrophils

Purified human neutrophils were prepared as described in 2.2.2

3.2.3 NF-κB activation

For these investigations, neutrophils were suspended in RPMI 1640 tissue culture medium (Highveld Biological, RSA) supplemented with 0.5% human serum albumin (HSA). Following 10 minutes of preincubation at 37°C, pneumolysin (8.37 and 41.75 ng/ml), metal (25 μM) or an equal volume of RPMI 1640 (control system) was added to the cells which were then incubated for 15 or 30 minutes at 37°C. The final volume in each tube was 1 ml containing $5x10^6$ cells. Following incubation, detection of NF-κB nuclear translocation was determined as described previously (Staal *et al*, 1995; Jimenez *et al*, 2000), with slight modifications. Briefly, cells were harvested and resuspended in 0.4 ml buffer (10 mM HEPES / 10mM KCl / 2 mM $MgCl_2$ / 1 mM DTT / 0.1 mM EDTA / 0.2 mM NaF / 0.2 mM Na_3VO_4) supplemented with the protease inhibitors 1 mg/L leupeptin and 0.4 mM PMSF, and placed on ice. After 15 minutes,
25 μl 10% Igepal CA-630 was added and the cells vortexed for 15 seconds and pelleted by centrifugation. Pellets containing the nuclear proteins were resuspended in buffer (50 mM HEPES / 50 mM KCl / 300 mM NaCl / 0.1 mM EDTA / 1 mM DTT / 10% glycerol / 0.2 mM NaF / 0.2 mM Na_3VO_4) supplemented with 0.1 mM PMSF and incubated on ice on a rotating platform for 20 minutes. After centrifugation for 5 minutes at 4°C, supernatants were collected and protein determinations performed.

For the electrophoretic mobility shift assay (EMSA), 7 μg of nuclear protein extract was incubated with ^{32}P-radiolabelled NF-κB-specific oligonucleotide (Amersham Biosciences UK Ltd, Amersham, UK) for 20 minutes at room temperature. Binding of NF-κB nuclear proteins to the oligonucleotide results in a retardation ("shift") of the electromobility on a 5% nondenaturating polyacrylamide gel. These shifts were visualized by phosphor-imaging using the Personal Molecular Imager FX and software from BIO-RAD Laboratories, Inc. Specificity of NF-κB DNA binding was ascertained by competition with excess unlabelled oligonucleotide, resulting in disappearance of NF-κB complexes, and results are shown as either the mean percentage counts/mm^2 of the pneumolysin/metal-free control system, or as the complete phosphor-images for representative experiments.

Additional experiments were performed to investigate the effects of the following on pneumolysin-mediated activation of NF-κB in neutrophils: i) inclusion of the extracellular Ca^{2+}-chelating agent EGTA (10 mM, final) in the cell-suspending medium; ii) the effects of pretreatment of the cells for 5 minutes with the omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA, 5 and 10 μg/ml, final).

3.2.4 Interleukin-8

3.2.4.1 Production of IL-8

To determine if exposure to metals would lead to increased synthesis of IL-8, neutrophils were preincubated for 10 minutes at 37°C in HSA (0.5%) supplemented RPMI 1640, followed by the addition of the different metals at a concentration of 25 μM. The tubes, containing $2x10^6$ cells in a final volume of 1 ml, were then incubated for 6hours at 37°C. Total and extracellular IL-8 was measured using the Bio-Plex suspension array system (see below), with the beads coated with anti IL-8 antibody. Total IL-8 was measured in the lysates of neutrophils that had been treated with 0.01% lysophosphatidylcholine, followed by centrifugation at 300g for 5 minutes to remove cellular debris, while extracellular cytokine was measured in cell-free supernatants, from which the cells had been removed by centrifugation.

3.2.4.2 Immunological reactivity of IL-8

To determine if exposure to metals would cause any changes in the immunological reactivity of IL-8 (human recombinant IL-8, Sigma-Aldrich), the cytokine, at a concentration of 1.25 μg/ml, was exposed to the metals (concentration 25 μM) for 3 minutes, after which the mixture was diluted 1:100 with HBSS (final concentration of IL-8, 12.5 ng/ml) and the concentration of IL-8 was determined in all systems, with unexposed IL-8 as control, using the Bio-Plex suspension array system.

3.2.5 Phosphorylated proteins

Cell lysates were prepared according to the instruction manual using the reagents supplied for phosphoprotein determination in the Bio-Plex cell lysis kit. Neutrophils at a concentration of $2x10^6$/ml were preincubated for 10 minutes at 37°C. After exposure to the metals for 3 minutes, the reaction was stopped by adding ice-cold wash buffer. Lysate solution was added and after freeze-thawing the lysate once, the samples were centrifuged at 4,500rpm for 20 minutes at 4°C. Supernatants were collected and phosphorylated proteins determined using the Bio-Plex suspension array system, the beads being coated with antibodies directed against the phosphorylated proteins of interest. A combination of phosphorylated-p38 MAPK, -IκBα, -JNK, -ERK1&2 (all of which are proteins implicated in the signalling pathways leading to the activation of NF-κB), STAT3, and ATF2 (transcription factors cooperating with NF-κB) was used. In this assay, the positive controls are extracts of HeLa cells, activated with specific activators for each enzyme/transcription factor (phosphoprotein), while untreated HeLa cells function as the background and/or negative control, as shown in table 3.1 (page 79). The optimal exposure time of the cells to the metals was established by exposing the neutrophils to Co^{2+}, Pt^{4+}, V^{3+}, and V^{5+} for 1, 3, 5 and 10 minutes followed by determination of the concentration of phosphorylated proteins.

Table 3.1

Lysates provided with the Bio-Plex assays:

Lysate	Phospho-specific target
EGF-Treated HEK 293	ERK1, ERK2, ERK1&2
EGF-Treated HeLa	EGFR
IFN-α-Treated HeLa	STAT3
TNF-α-Treated HeLa	IκB-α
UV-Treated HEK293	ATF-2, JNK, p38 MAPK
Untreated HeLa	Background/ negative control

The Bio-Plex array system utilizes the Luminex^{R}xMAP^{R}Multiplex technology to permit simultaneous detection and quantitation of up to 100 different analytes in a single microplate well. The system uses a liquid suspension array of 5.5 μm beads, each internally dyed with different ratios of two spectrally distinct fluorophores to assign it a unique spectral address. Each set of beads is conjugated with a different capture molecule, which binds to a target analyte. In the case of IL-8 and the phosphorylated proteins, the capture molecules are antibodies. This is followed by binding with biotinylated detection antibody and a reporter molecule, streptavidin-PE. The plate is then drawn into the Bio-Plex array reader, and precision fluidics align the beads in a single file through a flow cell where two lasers excite the beads individually. The red classification laser excites the dyes in each bead, identifying its spectral address, while the green reporter laser excites the reporter molecule associated with the bead, which allows quantitation of the captured analyte. Bio-Plex Manager software records the fluorescent signals simultaneously for each bead, translating the signals into data. Cytokine-results are expressed as pg/ml, while phosphorylation of target proteins is expressed according to fluorescence intensity.

3.2.6 Expression and statistical analysis of results

The results of each series of experiments are presented as the mean values+/- standard errors of the mean (SEM), with the exception of some of the experiments for the determination of NF-κB nuclear translocation, were the actual images of the gels are shown. 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®, Graph Pad Software Inc., San Diego, CA) was used for analysis and *P* value of <0.05 was taken as significant.

3.3 Results

3.3.1 NF-κB activation

3.3.1.1 NF-κB activation in pneumolysin-treated neutrophils

Exposure of neutrophils to pneumolysin caused a time- and dosed-related activation of NF-κB, which was attenuated by inclusion of the Ca^{2+}-chelating agent, EGTA in the cell-suspending medium or by pretreatment of the cells with docosahexaenoic acid (DHA). Images of gels are shown in Figures 3.1 and 3.2 (pages 82 and 83). The mean values of the density, expressed as counts/mm^2 are shown in Table 3.2 (page 84). These results clearly demonstrate, that pneumolysin causes activation of NF-κB in human neutrophils and that the system developed is suitable for the measurement of nuclear translocation of NF-κB proteins in these cells.

3.3.1.2 Effects of the metals on NF-κB activation

Exposure of neutrophils to the metals did not result in a meaningful increase in nuclear NF-κB proteins. The values of the densities, determined from 5 different experiments, expressed as counts/mm^2, are shown in Table 3.3 (page85).

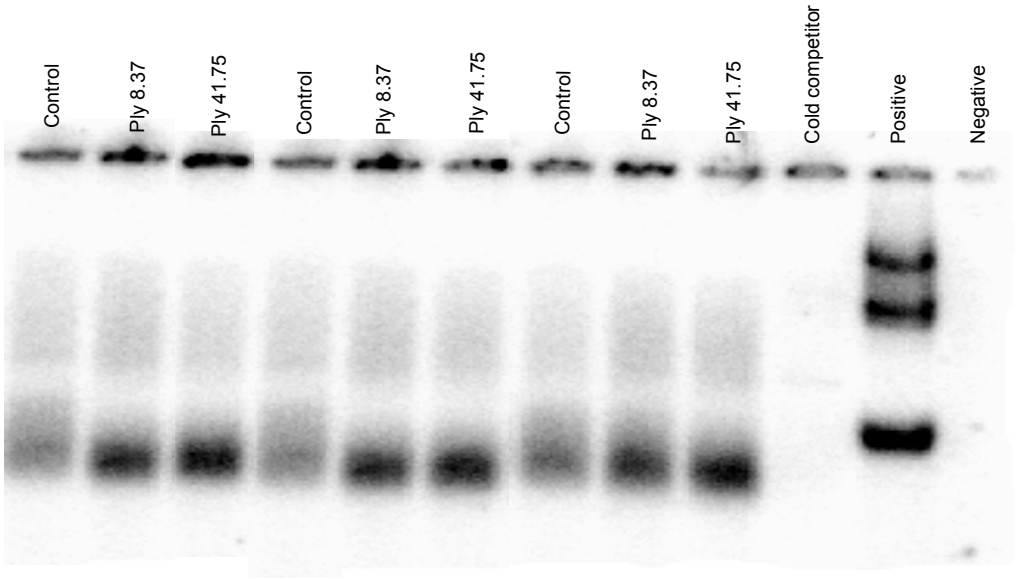

Figure 3.1

Phosphor-image showing the effects of pneumolysin on nuclear translocation of NF-κB in human neutrophils. The cells are from a single donor with triplicate determinations for each system (pneumolysin free control system, and systems treated with the toxin at concentrations of 8.37 ng/ml and 41.75 ng/ml).

Positive = HeLa cell nuclear extract

Negative = H_2O without any nuclear protein

Control = Neutrophils not exposed to pneumolysin

Cold competitor = positive sample previously incubated with non-radiolabelled NF-κB specific oligonucleotide.

This image is representative of 4 different experiments.

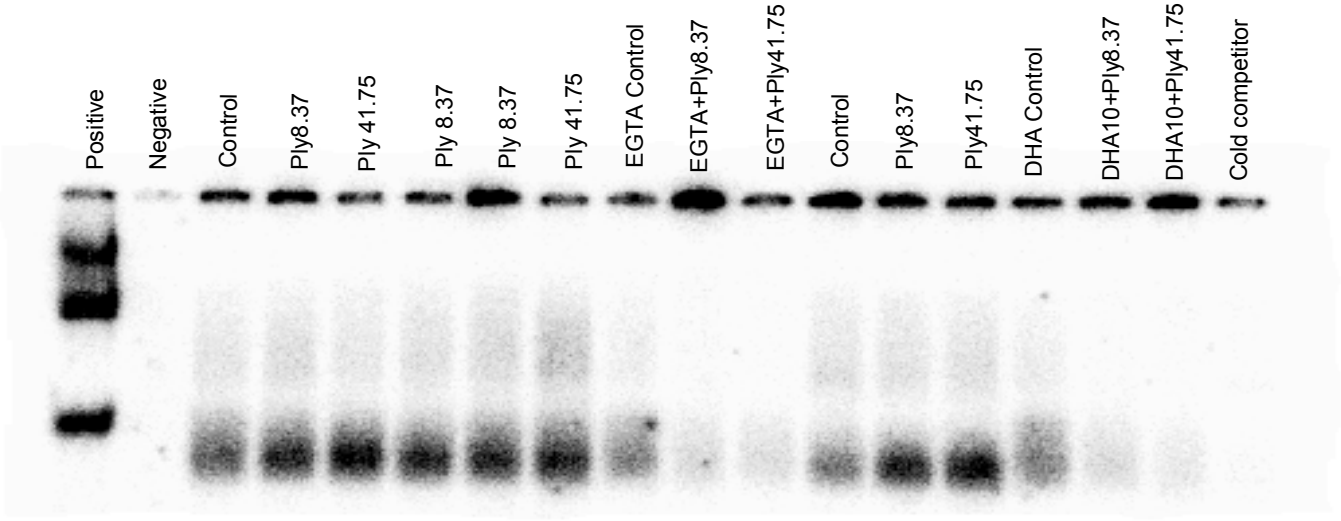

Figure 3.2
Phosphor-image showing the effects of pneumolysin (Ply 8.37 and 41.75 ng/ml) on nuclear translocation of NF-κB in human neutrophils in the absence and presence of 10 mM EGTA, or 10 μg/ml docosahexaenoic acid (DHA). The cells are from a single donor and the neutrophils were treated with pneumolysin for 15 min at 37°C in the absence and presence of EGTA or DHA and the nuclear extracts then analysed by electrophoretic mobility shift assay.
Positive = HeLa cell nuclear extract.
Negative = H_2O without any nuclear protein
Control = Neutrophils not exposed to pneumolysin
Cold competitor = sample previously incubated with non-radiolabelled NF-κB-specific oligonucleotide.
This image is representative of 4 different experiments.

Table 3.2

Mean values + SEM of the densities, expressed as counts/mm^2, of nuclear factor-κB translocated nuclear proteins as determined by the Personal Molecular Imager FX and software from BIO-RAD Laboratories. These are the results of 4 different experiments.

System	Density Counts/ mm^2
Positive	95 964 ± 2134
Negative (no nuclear extract)	12 619 ± 417
Control (neutrophils alone)	47 496 ± 2520
Neutrophils exposed to pneumolysin 8.37 ng/ml	71 652*± 2324
Neutrophils exposed to pneumolysin 41.75 ng/ml	80 305* ± 1266
EGTA control	49 782 ± 1936
EGTA + pneumolysin 8.37 ng/ml	23 273$^\Delta$ ± 1914
EGTA + pneumolysin 41.75 ng/ml	21 544$^\Delta$ ± 1580
DHA control	51 615 ± 1850
DHA + pneumolysin 8.37 ng/ml	22 020^o ± 1906
DHA + pneumolysin 41.75 ng/ml	18 287^o ± 1568
Cold competitor	11 035 ± 405

* $p < 0.05$ for comparison with the pneumolysin-free control system

Δ $p < 0.05$ for comparison with the corresponding pneumolysin- treated, EGTA-free system

o $p < 0.05$ for comparison with the corresponding pneumolysin-treated DHA-free system

Table 3.3

Effects of the metals on the activation of NF-κB in neutrophils.

Metal	NF-κB
Co^{2+}	101.22 ± 9.82*
Pd^{2+}	100.35 ± 13.7
Pt^{4+}	116.58 ± 8.15
V^{3+}	102.42 ± 11.03
V^{4+}	111.69 ± 19.06
V^{5+}	95.67 ± 17.90

* The results of 5 different experiments are expressed as the mean percentages ± SEM of the corresponding metal-free control systems. The absolute mean value ± SEM for the control system was 68 834 ± 10 624 counts/ mm^2.

3.3.2 IL-8

3.3.2.1 Production of IL-8

Human neutrophils were incubated with the different metals for 6 hours at 37°C, which had been previously determined as the optimal time for IL-8 production (Cockeran *et al*, 2002). As shown in Figures 3.3 and 3.4 (pages 86 and 87), no significant changes in either total or extracellular concentrations of IL-8 were detected following exposure of neutrophils to any of the metals when compared to the control. These observations are in agreement with the failure of the metals to activate NF-κB.

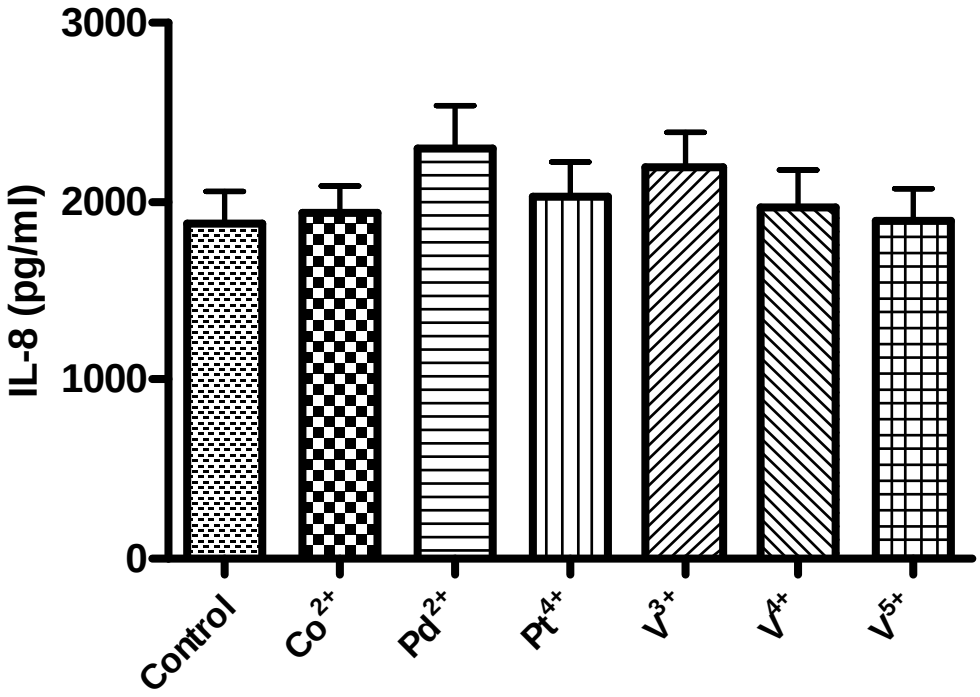

Figure 3.3

Effects of exposure to Co^{2+}, Pd^{2+}, Pt^{4+}, V^{3+}, V^{4+}, and V^{5+} on the production of IL-8 (measured in cell-free supernatants) by human neutrophils. Results are expressed as the mean values ± SEM of 8 experiments.

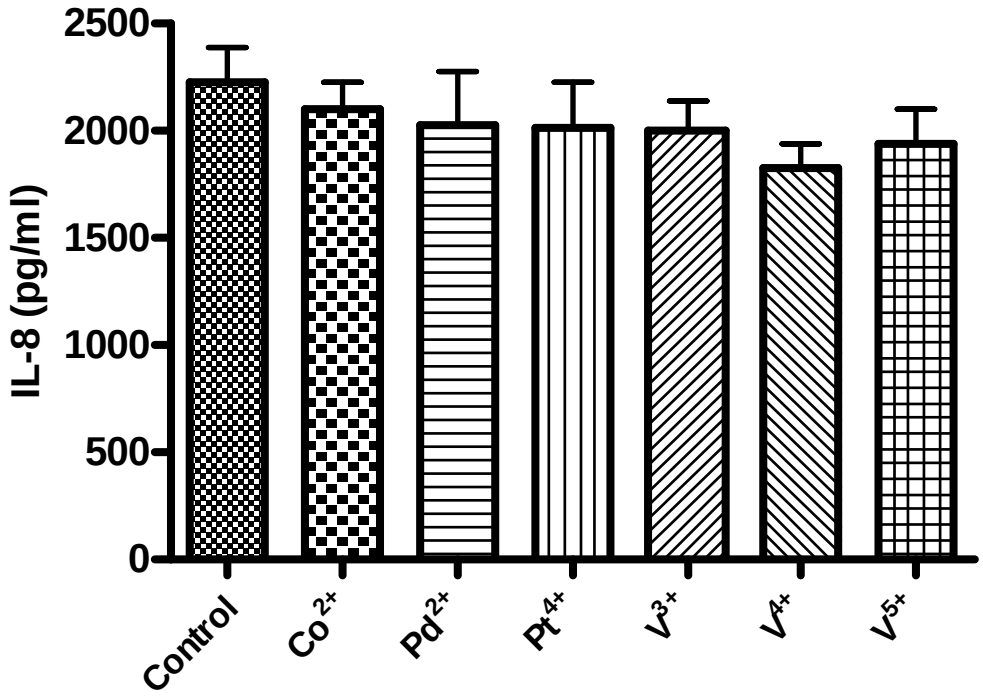

Figure 3.4

Effects of exposure to Co^{2+}, Pd^{2+}, Pt^{4+}, V^{3+}, V^{4+}, and V^{5+} on the production of IL-8 by human neutrophils. The results are the total (extracellular and intracellular) concentrations of IL-8 and are the mean values ± SEM of 8 experiments.

3.3.2.2 Immunological reactivity of IL-8

As shown in Table 3.4, exposure of IL-8 (1.25 μg/ml) to the metals (25 μM) caused no significant change in the reactivity of the chemokine. This clearly demonstrates that the metals do not affect the immunoreactivity of IL-8 in the Bio-Plex assay system.

Table 3.4

Effects of pre-treatment with Co^{2+}, Pd^{2+}, Pt^{4+}, V^{2+}, V^{3+}, V^{4+}, and V^{5+} (all at 25μM) on the immunological reactivity of IL-8.

System	Mean concentration of IL-8 (pg/ml)
IL-8 only	17 617 ± 2 753*
IL-8 + Co^{2+}	16 611 ± 1 920
Il-8 + Pd^{2+}	14 883 ± 2 050
IL-8 + Pt^{4+}	22 663 ± 1 710
IL-8 + V^{2+}	19 465 ± 2 560
IL-8 + V^{3+}	18 255 ± 1 8 62
IL-8 + V^{4+}	24 938 ± 2 240
IL-8 + V^{5+}	19 892 ± 1 790

The results of 5 experiments are expressed as the mean IL-8 concentrations ± SEM as determined with the Bio-Plex suspension array system.

***** Although used at a final concentration of 12 500 pg/ml, the values detected by the Bio-Plex assay system were somewhat higher (17 617 pg/ml in the control system). This may reflect a higher concentration of IL-8 than that reported by the manufacturer (Sigma) in the stock solution, and/or the high sensitivity of the Bio-Plex assay system.

3.3.3 Phosphorylated proteins

Time course experiments determined an optimal exposure time of neutrophils to the metals of 3 minutes. Of the phosphoproteins tested, phosphorylated p38 MAPK and phosphorylated IκB-α were present at the highest concentrations in the cytoplasm (Figure 3.5, page 90). As shown in Tables 3.5 and 3.6 (pages 91 and 92) exposure of the cells to the metals was not accompanied by detectable alterations in the levels of the phosphorylated enzymes / transcription factors.

These findings again confirm that the metals do not, either directly by interaction with the cell membrane, nor indirectly by increasing oxidative stress, initiate the phosphorylation of proteins involved in the NF-κB signalling pathways, nor do they cause phosphorylation of other transcription factors which act in unison with NF-κB.

3.3.4 Cellular ATP levels

The final concentration of each metal was 0.25μM, which was not cytotoxic to neutrophils as determined previously. The ATP levels of control neutrophils and those exposed to Pd^{2+}, Pt^{4+} were 32 ± 1, 35 ± 5, and 30 ± 2 nmol/10^7 cells, respectively (Theron *et al,* 2004). Experiments with cobalt resulted in 47 ± 3 for control neutrophils and 47± 2 nmol ATP/10^7 cells for metal-exposed neutrophils (Ramafi *et al,* 2004).

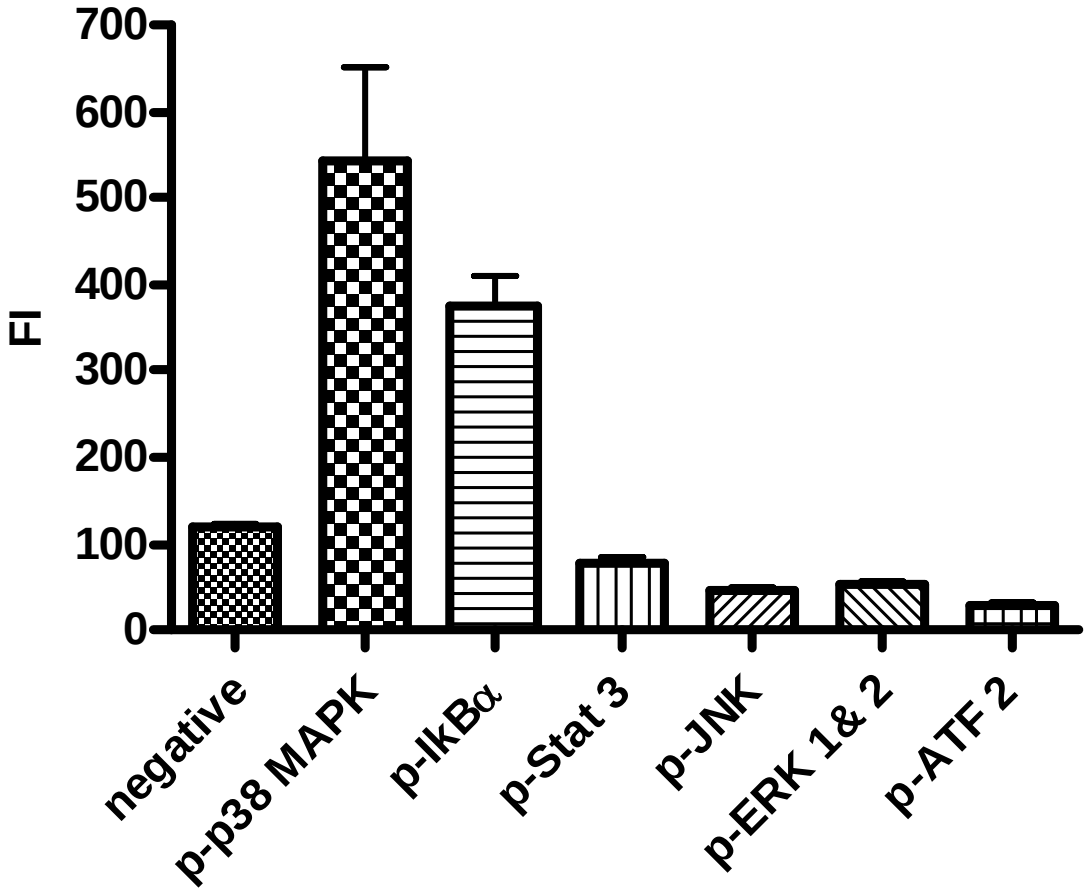

Figure 3.5

Concentrations of phosphorylated proteins in the cytosol of unstimulated human neutrophils in comparison to the cell-free, phosphorylation-free background system, which is referred to in the result section as the negative control system. The results are expressed as fluorescence intensity (FI) and are the mean values ± SEM from 4 different experiments.

Table 3.5

Phosphorylation of cytosolic proteins, involved in NF-κB signalling pathways, in unexposed and metal-exposed human neutrophils.

System	p-p38MAPK FI	p-IκB FI	p-JNK FI	p-Erk 1&2 FI
Positive control	2 052 ± 176	9 719 ± 555	2 072± 159	6 270 ± 149
Negative control	74 ± 2.1	198 ± 11	164 ± 45	250 ± 8.0
Unstimulated neutrophils	543 ± 107	375 ± 35	46 ± 3.7	54 ± 1.2
Neutrophils exposed to Co^{2+}	545 ± 50	386 ± 52	39 ± 3.6	52 ± 4.0
Neutrophils exposed to Pd^{2+}	435 ± 37	374 ± 48	46 ± 2.7	56 ± 1.7
Neutrophils exposed to Pt^{4+}	416 ± 47	364 ± 37	40 ± 0.4	54 ± 2.9
Neutrophils exposed to V^{3+}	496 ± 36	350 ± 31	37 ± 1.1	53 ± 3.6
Neutrophils exposed to V^{5+}	353 ± 67	311 ± 26	38 ± 3.1	55 ± 3.9

The results are expressed as fluorescence intensity (FI), and are the mean values ± SEM of four different experiments.

Table 3.6

Phosphorylation of the cytosolic transcription factors STAT3 and ATF2 in metal-treated and metal-untreated neutrophils.

System	p-STAT 3 FI	p-ATF 2 FI
Positive control	1 757 ± 138	1 394 ± 202
Negative control	120 ± 3.0	249 ± 34
Unstimulated neutrophils	79 ± 7.3	29 ± 2.7
Neutrophils exposed to Co^{2+}	72 ± 1.6	26 ± 2.0
Neutrophils exposed to Pd^{2+}	73 ± 3.1	26 ± 1.1
Neutrophils exposed to Pt^{4+}	72 ± 1.7	26 ± 1.0
Neutrophils exposed to V^{3+}	67 ± 0.3	26 ± 2.6
Neutrophils exposed to V^{5+}	76 ± 3.3	22 ± 0.5

The results are expressed as fluorescence intensity (FI), and are the mean values ± SEM from 4 different experiments.

3.4 Discussion

The first phase of the laboratory research described in this chapter was focussed on the development of an electrophoretic mobility shift assay (EMSA) for the detection of nuclear translocation of NF-κB in isolated human neutrophils. Based on previous reports that sustained elevations of cytosolic Ca^{2+} result in efficient activation of NF-κB in various cell types, including neutrophils (Dolmetsch *et al,* 1997; Bekay *et al*, 2003) the pore-forming pneumococcal toxin, pneumolysin, which causes influx of Ca^{2+} into neutrophils (Cockeran *et al.* 2001b), was identified as being a potential activator of NF-κB in these cells. The use of pneumolysin presented two advantages. Firstly, the pneumococcal toxin has been a research focus of the Department of Immunology, University of Pretoria, for several years, not only because of its involvement in the immunopathogenesis of invasive pneumococcal disease, but also because it represents an attractive target for drug and vaccine design. Secondly, the effects of pneumolysin on NF-κB activation in neutrophils have not been described previously, and thirdly, the effects of the various heavy metals (Co^{2+}, Pd^{2+}, Pt^{4+}, V^{2+-5+}) on the biological activities of pneumolysin represents a component of the studies presented in Chapter 4.

Exposure of neutrophils to low, sub-cytolytic, pathologically-relevant concentrations of pneumolysin (Spreer *et al.* 2003) resulted in significant, dose-related activation of NF-κB as determined by EMSA. The involvement of Ca^{2+} influx in pneumolysin-mediated activation of NF-κB was supported by observations that nuclear translocation of the transcription factor in toxin-activated cells was attenuated by inclusion of the Ca^{2+}-chelating agent, EGTA, in the cell-suspending medium, or by treatment of the cells with docosahexaenoic acid. Although not included in this chapter, treatment of the cells with the NADPH oxidase inhibitor, diphenylene iodonium chloride (5 μM) did not affect pneumolysin-mediated activation of NF-κB, seemingly excluding the involvement of reactive oxidant species in this process. Likewise, it has been described that the activation of NF-κB which accompanies exposure of neutrophils to opsonized *Staphylococcus aureus* was not dependent on

oxidants generated during immune adherence/phagocytosis (Vollebregt *et al.* 1998).

The second phase of the study was focussed on the ability of the metals to: i) initiate activation/translocation of NF-κB in neutrophils, as well as synthesis of IL-8; ii) activate cytosolic signalling pathways which activate NF-κB; and iii) activate other transcription factors which interact with NF-κB to optimize gene transcription. However, none of the metals induced phosphorylation and translocation of NF-κB, which was supported by observations that the metals also failed to affect the production of IL-8 by these cells. Not surprisingly, in view of the NF-κB and IL-8 results, none of the metals affected either the activities of the various phospho-enzymes involved in cytosolic signalling cascades which converge on NF-κB, or the other transcription factors which co-operate with NF-κB to promote optimal gene transcription.

The failure of the various test heavy metals to activate NF-κB in neutrophils contrasts with their ability to activate the transcription factor in other cell types, including epithelial cells (Goebeler *et al.* 1995; Sultana *et al.* 1955; Jiménez *et al.* 2000), macrophages (Catelas *et al.* 2003), synoviocytes (Niki *et al.* 2003), and monocytes (Lewis *et al.* 2003). These differences may be related to the relatively short exposure times of the cells to the metals used in the current study during which no cytotoxicity was evident, according to the observed absence of effects of the metals in cellular ATP levels. Possibly, with longer exposure times, activation of NF-κB may have been detectable. If this were to occur, however, it is likely to be secondary to cytotoxicity, as opposed to a rapidly occurring activation of NF-κB.

The laboratory research presented in this chapter was designed with the specific objective of investigating the effects of a single exposure of isolated human neutrophils to the individual metals in specific oxidation states over a relatively short time course on the activation of NF-κB. Such a study design is clearly necessary to identify the possible pro-inflammatory

interactions of the individual metals with the cells. I do concede, however, that this experimental design may not be representative of the pathophysiological setting in which cells of the innate immune system in the airways, including neutrophils, may undergo sustained exposure to the metals over a prolonged time course. Furthermore, in this scenario it is highly improbable that exposure to a single metal will occur, but rather to several metals in combination with other environmental/occupational toxins such as gases, particulate material, and bacterial endotoxins, as well as components of cigarette smoke. In addition, the behaviour of neutrophils in the airways may be affected indirectly as a consequence of exposure to chemokines/cytokines, as well as other activators, released from different cell types, such as epithelial cells, on exposure to the test metals.

Irrespective of these possible limitations, the results presented in the current chapter clearly demonstrate that exposure of isolated neutrophils to the test metals is not accompanied by activation of NF-κB or synthesis of the proinflammatory chemokine, IL-8.