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Effects of air pollution-related heavy metals on the viability and inflammatory responses of human airway epithelial cells

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Running title：Metals in air affect airway epithelial cells

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Abstract

Various metals produced from human activity are ubiquitously detected in ambient air. The metals may lead to induction and/or exacerbation of respiratory diseases, but the significant metals and factors contributing to such diseases have not been identified. To compare the effects of each metal and different oxidation states of metals on human airway, we examined the viability and production of interleukin (IL)-6 and IL-8 using BEAS-2B cell line, derived from human airway epithelial cells. Airway epithelial cells were exposed to Mn (+2), V (+4, +5), Cr (+3, +6), Zn (+2), Ni (+2), and Pb (+2) at a concentration of 0.5, 5, 50, or 500 μM for 24 h. Mn and V decreased the cell viability in a concentration dependent manner, and V (+5) tended to have a greater effect than V (+4). Cr decreased the cell viability, and Cr (+6) at concentrations of 50 and 500 μM was more toxic than Cr (+3). Zn at a concentration of 500 μM greatly decreased the cell viability, whereas Ni at the same concentration increased it. Pb produced fewer changes. Mn and Ni at a concentration of 500 μM induced the significant production of IL-6 and IL-8. However, most of the metals including V (+4,

+5), Cr (+3, +6), Zn, and Pb inhibited the production of both IL-6 and IL-8. The present results indicates that various heavy metals have different effects on toxicity and the pro-inflammatory responses of airway epithelial cells, and those influences also depend on the oxidation states of the metals.

Key Words: airway epithelial cells, heavy metals, oxidation states of metals, viability**,** pro-inflammatory responses

Introduction

Particulate matter (PM) in the atmosphere is composed of solid and liquid materials which contain / elemental carbon (EC), organic carbon (OC), inorganic salts and metals. For example, diesel exhaust particles (DEPs) have a carbon core on which organic chemical components including polycyclic aromatic hydrocarbons (PAHs) and semi-volatile organic compounds (SVOCs), sulfate and nitrate ions, and heavy metals are adsorbed.[1, 2] Human exposure to these constituents of PM occurs through inhalation, which may lead to the induction and/or exacerbation of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and lung cancer.[3] However, it is not clear which metal compositions contribute to respiratory diseases because PM is an aggregate of a particle and a large number of chemicals and metal materials, and because the compositions of PM can differ by time and place.

Previous experimental studies have indicated that not only the insoluble fraction but also the soluble fraction in PM contributes to respiratory diseases. In an *in vitro* study, Knaapen et al.[4] have suggested that the soluble fraction as well as the

insoluble particle fraction induces cellular DNA damage in human alveolar epithelial cells (A549). An *in vivo* study by Adamson et al.[5] has demonstrated that instilling the soluble fraction to mouse lung produces inflammatory changes and lung injury. The water-soluble fraction in PM may contain various metals, and the metals can be one of the risk factors which contribute to the development/exacerbation of respiratory diseases.

Various metals emitted from human activity are ubiquitously detected in Earth's atmosphere.[6, 7] The metal oxide particles are produced from the combustion of fossil fuels and metallurgical activities. They are emitted as fly ash into the atmosphere, and may be partly transformed into soluble metals when they co-exist with sulfate and nitrate ions.[8, 9] The solubility of metals depends on the pH and combustion conditions such as temperature and added reagents.[10, 11] Epidemiological studies have shown that increases in the ambient nickel (Ni) and vanadium (V) concentrations are significantly associated with an increased probability of wheezing in young children.[12] Decrements in lung function indices associated with increasing concentrations of zinc (Zn) and iron

(Fe) have been observed in COPD subjects.[13] Increases in ambient Zn have been associated with increases in asthma emergency department visits and hospital admissions among children.[14] Hexavalent chromium (Cr^{+6}) has been generally known to cause lung cancer.[15] Laden et al.[16] have reported that Ni, lead (Pb), and sulfur in the atmosphere may influence total mortality. However, it has not been fully clarified which metals generated from different sources contribute to respiratory health effects.

In this study, we focused on the effects of relatively soluble metals emitted from human activities on human airway epithelial cells. Specially, we compared the effects of metals including manganese (Mn), V, Cr, Zn, Ni, and Pb and the effects of different oxidation states of metals on cellular viability and pro-inflammatory responses. The critical point of the study is to compare different metals and different oxidation states under the same condition.

Materials and methods

Cell culture

The BEAS-2B cell line, derived from human bronchial epithelial cells transformed by an adenovirus 12-SV40 hybrid virus, was purchased from European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Airway epithelial cells were seeded in 96-well or 12-well collagen I-coated plates and incubated for 72 h to reach semi-confluence in the serum-free medium LHC-9 (Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Experimental protocol

Metals (Sigma Chemical, St. Louis, MO) including Mn, V, Cr, Zn, Ni, and Pb (Purity; ≥99.6%) were used in the experiments. Mn was prepared in one oxidation state: Mn^{+2} ($MnSO_4$・n H_2O). V was prepared in two oxidation states: V^{+4} ($VOSO_4$・n H_2O) and V^{+5} (V_2O_5). Cr was prepared in two oxidation states: Cr^{+3} (Cr $(NO_3)_3$・$9H_2O$) and Cr^{+6} ($K_2Cr_2O_7$). Zn was prepared in one oxidation state: Zn^{+2} ($ZnSO_4$・$7H_2O$). Ni was prepared in one oxidation state: Ni^{+2} ($NiSO_4$・$6H_2O$). Pb was prepared in one oxidation state: Pb^{+2} (Pb $(NO_3)_2$). These metals were prepared in sterilized ultrapure water and/or

medium.

After airway epithelial cells grew to semi-confluence in LHC-9, the cells were exposed to metals at a concentration of 0, 0.5, 5, 50, or 500 μM for 24 h. The cell viability and the release of interleukin (IL)-6 and IL-8 into the culture supernatants were evaluated by WST-1 assay and enzyme-linked immunosorbent assay (ELISA), respectively.

The critical point of this study was to compare the effects of different metals under the same experimental condition. Pb^{2+} and Ni^{2+} are known to cause low toxicity, whereas Cr^{6+} has high toxicity according to the previous reports.[17, 18, 19] The exposure time and doses selected for this study were based on the variety of toxicities previously reported for the determination of cell viability and pro-inflammatory responses. Previous studies also have used similar doses and time points as those of the present study to investigate the effects of each metal including Mn^{+2}, V^{+4}, V^{+5}, Cr^{+6}, Zn^{+2}, Ni^{+2} on airway epithelial cells (Table 1 and 2).

Cell viability

Cell viability was measured by WST-1 assay using the Premix WST-1 Cell Proliferation Assay System (TaKaRa Bio, Shiga, Japan). In brief, WST-1 reagent was added to each well of 96-well plate and mixed by gently rocking the plate. Airway epithelial cells were incubated with WST-1 reagent at 37°C for 3 h. After incubation, absorbance was measured on an iMarkMicroplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA) with the wavelength at 450 nm and a reference wavelength at 630 nm. Results are expressed as the percentage of viable cells compared to untreated cells (0 μM).

Quantitation of inflammatory proteins in the culture supernatants

After exposure to metals, the medium was harvested and centrifuged at 300×*g* for 5 min to remove floating cells. The final supernatants were stored at −80°C until analysis. The levels of IL-6 and IL-8 (Thermo Scientific, Waltham, MA, USA) in the culture medium were measured by ELISA according to the manufacturer's instructions.

Absorbance was measured on the iMark Microplate Absorbance Reader with the wavelength at 450 nm and a reference wavelength at 550 nm. The detection limits of the IL-6 and IL-8 assay were less than 0.8–1.2 pg/mL and 0.5–2.6 pg/mL, respectively.

Statistical analysis

Data are represented as mean ± standard error of the mean (S.E.M.) for each experimental group (n=3–4). Differences among groups were analyzed using the Dunnett multiple comparison test (Excel Statistics 2010, Social Survey Research Information Co. Ltd., Tokyo, Japan). A p value < 0.05 was considered to indicate a significant difference.

Results

Effects of metals on the viability of airway epithelial cells

We investigated the effects of metals on the cellular viability of airway epithelial cells after exposure to each metal for 24 h (Fig. 1). Mn^{+2}, V^{+4} and V^{+5}

decreased the cell viability in a concentration-dependent manner, and V^{+5} tended to have a greater effect on cell viability than V^{+4}. Cr^{+6} and Cr^{+3} decreased the cell viability, and Cr^{+6} at concentrations of 50 and 500 μM was more toxic than Cr^{+3}. Zn^{+2} at a concentration of 500 μM produced a dramatic decrease in cell viability. In contrast, Ni^{+2} at a concentration of 500 μM increased cell viability, whereas Pb^{+2} showed less change. The order of toxicity based on TC50 values (concentration that reduces cell viability to 50%) was Mn^{+2} (3.0 μM) > Cr^{+6} (7.5 μM) > V^{+5} (36.3 μM) > V^{+4} (86.8 μM) > Zn^{+2} (427.6 μM). Ni^{+2}, Pb^{+2}, and Cr^{+3} showed low or no inhibition of cellular viability, and therefore their TC50 values could not be calculated.

Effects of the metals on cytokine production from airway epithelial cells

Airway epithelial cells, which have important roles in the physical barrier and immunological responses against xenobiotics, are a source of cytokines. We investigated the effects of the metals on the pro-inflammatory responses of human airway epithelial cells, and we examined the productions of IL-6 (Fig. 2) and IL-8 (Fig.

3) after exposure to each metal for 24 h.

Mn^{+2} at a concentration of 500 μM elevated the IL-6 release, but the levels of IL-6 at the concentrations of 0.5 and 5 μM were significantly lower than the level at 0 μM. V^{+4} and V^{+5} at a concentration of 0.5 μM decreased the release of IL-6 compared to 0 μM, whereas the levels of IL-6 recovered after exposure at 5 μM, and then the levels at concentrations of 50 and 500 μM decreased again. V^{+5} tended to be more influential than V^{+4}. Cr^{+6} decreased the release of IL-6 in a concentration-dependent manner. Cr^{+3} depressed the release of IL-6 except for exposure at a concentration of 50 μM. Zn^{+2} concentration-dependently decreased the IL-6 release, and the IL-6 level at the 500 μM exposure was below the detection limit. In contrast, Ni^{+2} at a concentration of 500 μM markedly increased the level, whereas Ni^{+2} at concentrations of 0.5, 5, and 50 μM lowered the level of IL-6. Pb^{+2} inhibited the IL-6 release at all concentrations.

In the results of IL-8, the IL-8 protein release showed a similar tendency to that of IL-6 release. Mn^{+2} at a concentration of 500 μM greatly elevated the IL-8 release. V^{+4} and V^{+5} decreased the release of IL-8 in a roughly concentration-dependent manner.

V^{+5} tended to be more influential than V^{+4}. Cr^{+3} and Cr^{+6} decreased the release of IL-8 at all concentrations. The effects of Cr^{+6} at concentrations of 5, 50 and 500 μM were stronger than those of Cr^{+3}. Zn^{+2} at concentrations of 0.5, 5, and, 50 μM decreased the levels of IL-8, and the level at a concentration of 500 μM exposure showed a dramatic inhibition. Ni^{+2} at a concentration of 500 μM produced an extreme elevation of IL-8 release; the levels of IL-8 at a concentration of 500 μM were 14-fold higher than those at 0 μM. Pb^{+2} at concentrations of 0.5 and 50 μM decreased the IL-8 release.

Mn^{+2} and Ni^{+2} elevated the IL-6 and IL-8 protein releases at 500 μM, at which concentration Mn^{+2} showed inhibition of viability and Ni^{+2} demonstrated no toxicity. The other metals significantly decreased IL-6 and IL-8 protein release.

Discussion

We found that exposing human airway epithelial cells to some metals affected the cell viability and changed pro-inflammatory responses via the expression of IL-6 and IL-8. The responses of these biomarkers showed differing profiles when exposed to

a variety of metals and oxidation states.

In this study, the order of toxicity based on TC50 values was Mn^{+2} (3.0 μM) > Cr^{+6} (7.5 μM) > V^{+5} (36.3 μM) > V^{+4} (86.8 μM) > Zn^{+2} (427.6 μM). Ni^{+2}, Pb^{+2}, and Cr^{+3} showed low or no inhibition of cellular viability. It is especially notable that Mn^{+2} as well as Cr^{6+} at low concentrations showed greater toxicity against airway epithelial cells cultured in serum-free LHC-9 medium. Although Mn^{+2} has been known to induce the lung inflammation in experimental investigation[20], comparative studies among various metals are little performed. Our results clarified that Mn^{+2} is a highly toxic metal against airway cells, even among various metals. Riley et al.[18] have indicated that the ranking of metal toxicity is V^{+4} (VCl_4) > Zn^{+2} ($ZnCl_2$) > Cu^{+2} > Ni^{+2} ($NiCl_2$) > Fe^{+2} in a rat lung epithelial cell line (RLE-6TN). In addition, the order of cytotoxicity in BEAS-2B cultured in keratinocyte growth medium has been Cd^{+2} > Cr^{+6} (CrO_3) > Pt^{+4} > Pd^{+2} ($PdSO_4$)= Pt^{+2} > Ni^{+2} ($NiCl_2$) > Rh^{+3}.[19] Pascal and Tessier[21] have reported that Cr^{+6} ($K_2Cr_2O_7$) and Mn^{+2}, but not Ni^{+2}, are cytotoxic to BEAS-2B cultured in F-12 medium with 10% fetal bovine serum. It is difficult to compare the previous studies

with our results, because the culture conditions and metal complexes differ. However, the results on the ranking of metal toxicity based on TC50 values in the present study resembled those of previous studies.[18, 19, 21] These results suggest that relatively soluble metals in sulfate, nitrate, oxides, and dichromate form which may exist in the atmosphere[9, 22] have different cellular toxicities among a variety of metals and oxidation states of metals.

IL-6 and IL-8 are pro-inflammatory cytokines induced by environmental insults, and they play important roles in inflammation in the respiratory system by stimulating lymphocytes, inducing neutrophils recruitment and up-regulating mucin secretion.[23, 24, 25, 26] Our study focused on non-specific inflammatory responses and cell viability in airway epithelial cells rather than specific inflammatory responses by immune cells. Therefore IL-6 and IL-8 were measured as non-specific inflammatory response markers. In addition, in our past experiments, IL-6 and IL-8 released from BEAS-2B changed in response to some air pollutants such as Asian sand dust particles.[27] Moreover, the effect of these molecules *in vitro* correlated well with airway

inflammation after *in vivo* exposure by bronchoalveolar lavage.[28] In this study, Ni^{+2} and Mn^{+2} especially elevated the release of IL-6 and IL-8 among metals.

Interestingly, although Ni^{+2} at the high dose increased viability and release of IL-6/IL-8, Ni^{+2} at lower doses resulted in reduced viability and release of IL-6/IL-8. It has been reported that exposure to Ni^{+2} ($NiSO_4$) induces the secretion of IL-8 in airway epithelial cells[29], and that Ni^{+2} compounds induce oxidative stress.[30] Ni^{+2} at the high dose may cause pro-inflammatory responses via reactive oxygen species (ROS). Ni^{+2} at the high dose may also induce metallothionein which is an antioxidatant and a cytoprotective protein against metal toxicities.[31] It has been also reported that high levels of metallothionein in the nucleus of cells contributes to promoting cell proliferation.[32] In brief, increased pro-inflammatory responses at the high dose have possibility to occur via ROS. Moreover, increased viability may relate with the effects of metallothionein induced by Ni^{+2}. On the other hand, Ni^{+2} at lower doses may not produce ROS although the expression of metallothionein may be slightly induced in response to Ni^{+2}. Therefore, metallothionein as an antioxidative molecule may scavenge

ROS under control conditions, and Ni^{+2} at lower doses mainly inhibit the release of IL-6 as shown in the present study. Accordingly, the cellular events at the high dose can be different from those at the low doses.

Mn^{+2} at a high dose showed inhibition of viability and elevation of IL-6/IL-8 release. It has been reported that exposure to Mn^{+2} induces the secretion of IL-6 or IL-8 in airway epithelial cells[21], and that Mn^{+2} compounds induce oxidative stress.[33] Mn at a dose of 500 μM showed high toxicity against airway epithelial cells. After Mn induces pro-inflammatory protein in the early stages, cell death such as necrosis may happen.

The other metals significantly decreased IL-6 and IL-8 protein release. The inhibitory effect on IL-6 and IL-8 caused by V^{+4}, V^{+5}, Cr^{+6} and Zn^{+2} at high concentrations may be due to the cytotoxic effect. However, apart from the results obtained with high concentrations, the present findings are inconsistent with those of previous studies. Some studies have reported that Mn^{+2}, V^{+4}, V^{+5}, Cr^{+6}, Zn^{+2}, Ni^{+2} (and not Cr^{+3} and Pb^{+2}) induce IL-6 and IL-8 from airway epithelial cells (Tables 1 and 2). On the other hand, in this study, Cr^{+3} reduced cell viability to about 80% at all doses,

and depressed IL-6 release except for a concentration of 50 μM. Pb^{+2} decreased IL-6/IL-8 in some doses with no cytotoxicity. Although Cr^{+3} and Pb^{+2} are known to show low cellular toxicity,[17,34] there are few studies showing changes to pro-inflammatory cytokines in airway epithelial cells. The discrepancies appear to depend on the differences in cells, media, and metal complex forms. Veranth et al.[35] have suggested that the IL-6 response to V^{+4} ($VOSO_4$) treatments changes when the same cells, BEAS-2B, are grown in KGM or LHC-9 medium. In brief, BEAS-2B in LHC-9 has shown no response to V^{+4}, whereas BEAS-2B in KGM has produced IL-6. The Veranth group has noted that the method of cell passaging, and the exact growth factors in the media, are likely to affect both the populations of receptors on the cell surface and the intracellular signal transduction. Actually, in addition to the findings reported by Veranth et al.[35], unchanged response of IL-6 and IL-8 to Ni^{+2} and decreased response of IL-6 to Zn^{+2} have been also observed. Salnikow et al.[29], Carter et al.[36], and Jaspers et al.[37] have shown different IL-8 responses after Ni^{+2} exposure in different cells and media (Table 2). There have also been reports that the IL-6 release in RLE cultures

significantly decreases in response to Zn^{+2} exposure at a concentration of 100 μM, which causes < 20% cell death (Table 1; Riley et al.[18]). Further investigations are needed to understand the meanings and the mechanism by which metals decrease IL-6 and/or IL-8 release.

We investigated the effect of different oxidation states of V and Cr on human airway epithelial cells. V^{+4} and Cr^{+3} are known to be more stable compared with V^{+5} and Cr^{+6}, respectively. When humans inhale PM, airway epithelial cells may often encounter V^{+4} and Cr^{+3}. In the aqueous *in vitro* setting, V^{+5} and Cr^{+6} may partially change into chemical forms of V^{+4} and Cr^{+3}. In this study, even though they are the same element, different oxidation states of V and Cr have demonstrated different behaviors in biological reaction.

V in the atmosphere results from the combustion of residual fuel oil. It has been reported that most of the V spectra in the combustion of residual fuel oil closely resembles those of $VOSO_4$, and oxide, probably V_2O_5.[8] In present study, V^{+5} (V_2O_5) tended to be more influential than V^{+4} ($VOSO_4$). It has been reported that the toxicity

caused by the ingestion of V^{+5} (V_2O_5) is higher than the toxicity caused by V^{+4} ($VOSO_4$).[38] However, Pierce et al.[39] have reported that the intratracheal instillation of V^{+4} ($VOSO_4$) in rat induces a higher neutrophil influx in bronchoalveolar lavage than that of intratracheal instillation of V^{+5} (V_2O_5). They have suggested that V^{+5} (V_2O_5) would dissolve less quickly in surfactant of the lung. The toxicity of V compounds may differ by exposure routes (oral or intratracheal instillation) *in vivo* study. However, in an *in vitro* study using V^{+4} ($VOSO_4$) and V^{+5} (Na_3VO_4), Carter et al.[36] have indicated that V^{+4} ($VOSO_4$) and V^{+5} (Na_3VO_4) are equally potent in inducing the production of IL-6, and V^{+4} ($VOSO_4$) induces slightly higher levels of IL-8 than V^{+5} (Na_3VO_4) in normal human bronchial epithelial cells cultured in BEGM media. As mentioned above, the differences in oxidation state may also depend on the cell type, culture condition and metal complex. The impact of V compounds on pro-inflammatory reactions in the human airway has not been clear. Further investigations are needed to understand the different behaviors of V^{+4} and V^{+5} in biological reaction.

The oxidation states of Cr which exist in the atmospheric environment are Cr^{+3}

and Cr^{+6}. Chemical reactions between Cr^{+3} and Cr^{+6} would occur in the aqueous phase of PM. The unstable species Cr^{+6} reduce to Cr^{+3} under typical atmospheric conditions.[40] Previous studies have suggested that Cr^{+6} is more toxic than Cr^{+3} in multiple types of cells, and that Cr^{+6} causes cytotoxicity during the reduction of Cr^{+6} to Cr^{+3} in cells.[34] Accordingly, as V^{+4} and V^{+5}, or Cr^{+3} and Cr^{+6} show different responses, it is important to understand the effect of the differences in oxidation state on the airway as well as the differences in metal element.

Humans can be exposed to air pollutants containing various metals. However, in this test system, we have not examined combinations of metals. The synergistic or antagonistic relationships may occur depending on the metal transporter and cell signal transduction. Indeed, it has been reported that the simultaneous addition of iron in either ferric or ferrous form and nickel completely inhibit IL-8 production in the 1HAEo- cells.[29] That will be a subject for future analysis.

In addition to the immortalized BEAS-2B cell line used as an *in vitro* model, we may need studying sensitivity against each metal under conditions which are more

close to *in vivo* such as primary cells. Because BEAS-2B cells have inherent limitations in cell culture studies although BEAS-2B is one of cell lines used to evaluate biological responses induced by environmental pollutants. For examples, Mn-superoxide dismutase activity has been able to be lower in BEAS-2B cells than in primary cultures.[45] Moreover, BEAS-2B cells in two-dimensional systems fail to undergo mucociliary differentiation. Recently, polarized human airway epithelial cells in air-liquid interface (ALI) cultures are developing as a respiratory model; they enable mucociliary transport. [46, 47] The mucus on the apical side of airway epithelial cells protects from environmental stimuli. These characteristics may have impacts on metal toxicity as involves the production of reactive oxygen species. Accordingly, *in vitro* exposures using not only BEAS-2B cell lines but also primary cells and/or ALI cultures may enable us to compare with real human exposures adequately.

Conclusion

The present study obtained comparative data among metals. We have found

that exposure to various heavy metals results in differing cell toxicity and pro-inflammatory responses of airway epithelial cells, and these differences also depend on the oxidation states of the metals. The biological reaction of airway epithelial cells to metals in the atmosphere can lead to airway damage and the development/exacerbation of respiratory diseases.

It has been reported that ambient PM2.5 increases and/or decreases pro-inflammatory protein in airway epithelial cells. [48, 49, 50] The components of ambient PM2.5 including metals differ in place and time, which changes the effects on respiratory health. In brief, increased and/or decreased pro-inflammatory protein may depend on quantity of metals. Accordingly, this study contributes to elucidating mechanism by which air pollutants cause/inhibit pro-inflammatory responses in real exposed situation.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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Table 1. Literature evaluation of IL-6 responses against metals under various experimental conditions using airway epithelial cells.

Cells	Medium	Metal compounds	Oxidation state	Experimental concentrations	Period	Outcome of IL-6	Citation
SAEC	SAGM	?	Mn^{+2}	0.2, 2, 20, 200 μM	6 h	Increase (Protein)	Pascal and Tessier.[21]
BEAS-2B	LHC-9	$VOSO_4$	V^{+4}	100 μg/cm^2	20-22 h	**No change** (Protein)	Veranth et al.[35]
BEAS-2B	KGM	$VOSO_4$	V^{+4}	100 μg/cm^2	20-22 h	Increase (Protein)	Veranth et al.[35]
NHBE	BEGM	$VOSO_4$	V^{+4}	100, 500, 750 μM	2 h	Increase (Protein)	Carter et al.[36]
BEAS-2B	KGM	$VOSO_4$	V^{+4}	80 μg/mL	24 h	Increase (Protein)	Veranth et al.[44]
NHBE	BEGM	$NaVO_3$	V^{+5}	100, 500, 750 μM	2 h	Increase (Protein)	Carter et al.[36]
SAEC	SAGM	$K_2Cr_2O_7$	Cr^{+6}	0.2, 2, 20, 200 μM	6 h	Increase (Protein)	Pascal and Tessier.[21]
BEAS-2B	LHC-9	$K_2Cr_2O_7$	Cr^{+6}	5 μM	72 h	Increase (mRNA)	O'Hara et al.[42]
RLE-6TN	DME	$ZnCl_2$	Zn^{+2}	100, 1000 μM	24 h	**Decrease** (Protein)	Riley et al.[18]
NHBE	BEGM	$NiSO_4$	Ni^{+2}	100, 500, 750 μM	2 h	**No change** (Protein) data not shown	Carter et al.[36]

[Cells] SAEC: Normal human small airway epithelial cells, **NHBE**: Normal human bronchial epithelial cells, **RLE-6TN** : a rat type II alveolar epithelial cells **[Medium] SAGM**: Small airway growth medium supplemented with 30 μg/mL bovine pituitary extract, 0.5 μg/mL hydrocortisone, 0.5 mg/mL human

recombinant epidermal growth factor, 0.5 μg/mL epinephrine, 10 μg/mL transferring, 5 μg/mL insulin, 0.1 mg/mL retinoic acid, 6.5 mg/mL triiodothryonine, 50 μg/mL gentamicin, 50 mg/mL amphotericin, and 5% fatty acid-free bovine serum albumin, **KGM**: Keratinocyte growth medium is prepared from KBM basal media with additives, **BEGM**: Bronchial epithelial cell growth medium known as LHC-9 with modification, **DME**: DME media supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution.

Table 2. Literature evaluation of IL-8 responses against metals under various experimental conditions using airway epithelial cells.

Cells	Medium	Metal compounds	Oxidation state	Experimental concentrations	Period	Outcome of IL-8	Citation
SAEC	SAGM	?	Mn^{+2}	0.2, 2, 20, 200 μM	6 h	Increase (Protein)	Pascal and Tessier.[21]
NHBE	BEGM	$VOSO_4$	V^{+4}	100, 500, 750 μM	2 h	Increase (Protein)	Carter et al.[36]
Primary NHBE	BEGM	$VOSO_4$	V^{+4}	12.5, 25, 50 μM	24 h	Increase (Protein)	Jaspers et al.[37]
BEAS-2B	KBM	$VOSO_4$	V^{+4}	500 μM	20 min exposure and then sampled at 6 and 24 h	Increase (Protein)	Samet et al.[43]
NHBE	BEGM	$NaVO_3$	V^{+5}	100, 500, 750 μM	2 h	Increase (Protein)	Carter et al.[36]
BEAS-2B	KBM	$Cr_2(SO_4)_3$	Cr^{+3}	500 μM	20 min exposure and then sampled at 6 and 24 h	**No change** (Protein)	Samet et al.[43]
SAEC	SAGM	$K_2Cr_2O_7$	Cr^{+6}	0.2, 2, 20, 200 μM	6 h	Increase (Protein)	Pascal and Tessier.[21]
BEAS-2B	KBM	$ZnSO_4$	Zn^{+2}	500 μM	20 min exposure and then sampled at 6 and 24 h	Increase (Protein)	Samet et al.[43]
BEAS-2B	KGM	$ZnSO_4$	Zn^{+2}	15, 50 μM	12 h	Increase (Protein)	Kim et al.[41]

1HAEo^{-}	RPMI 1640	$NiSO_4$	Ni^{+2}	250 μM	8, 16, 24, 40, 48 h	Increase (Protein)	Salnikow et al.[29]
Primary NHBE	BEGM	$NiSO_4$	Ni^{+2}	12.5, 25, 50 μM	24 h	**No change** (Protein) data not shown	Jaspers et al.[37]
NHBE	BEGM	$NiSO_4$	Ni^{+2}	100, 500, 750 μM	2 h	**No change** (Protein) data not shown	Carter et al.[36]

[Cells] SAEC: Normal human small airway epithelial cells, **NHBE**: Normal human bronchial epithelial cells, **Primary NHBE**: Normal human bronchial epithelial cells obtained from healthy, nonsmoking adult volunteers, **1HAEo^{-}**: SV40-transformed normal human airway epithelial cells, **[Medium] SAGM:** Small airway growth medium supplemented with 30 μg/mL bovine pituitary extract, 0.5 μg/mL hydrocortisone, 0.5 mg/mL human recombinant epidermal growth factor, 0.5 μg/mL epinephrine, 10 μg/mL transferring, 5 μg/mL insulin, 0.1 mg/mL retinoic acid, 6.5 mg/mL triiodothryonine, 50 μg/mL gentamicin, 50 mg/mL amphotericin, and 5% fatty acid-free bovine serum albumin, **BEGM**: Bronchial epithelial cell growth medium known as LHC-9 with modification, **KBM**: Keratinocyte basal medium supplemented with 30 μg/mL bovine pituitary extract, 5 ng/mL human epidermal growth factor, 500 ng/mL hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 5 ng/mL insulin. Cells were replaced in KBM without supplements before experiments. **SAGM:** Small airway growth medium supplemented with 30 μg/mL bovine pituitary extract, 0.5 μg/mL hydrocortisone, 0.5 mg/mL human recombinant epidermal growth factor, 0.5 μg/mL epinephrine, 10 μg/mL transferring, 5 μg/mL insulin, 0.1 mg/mL retinoic acid, 6.5 mg/mL triiodothryonine, 50 μg/mL gentamicin, 50 mg/mL amphotericin, and 5% fatty acid-free bovine serum albumin, **KGM:** Keratinocyte growth medium, **RPMI1640**: Cells were changed to a serum-free/iron-free RPMI 1640 medium, after cells were grown in medium with Earle's modified salts containing 10% FCS, 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin.

Figure legends

Figure 1. Effects of metals on the viability of human airway epithelial cells. Cells were treated with the indicated concentrations of metals for 24 h. Cell viability was assessed by WST-1 assays. Data are presented as the percentage of the viability of the control (0 μM). Data are mean ± SEM of 3-4 individual cultures. $^{*}p<0.05$ versus 0 μM.

Figure 2. IL-6 production from airway epithelial cells in response to metals. The protein levels in the culture supernatant after exposure to metals for 24 h were measured by ELISA. Data are mean ± SEM of 3-4 individual cultures. $^{*}p<0.05$ versus 0 μM. ND: not detected.

Figure 3. IL-8 production from airway epithelial cells in response to metals. The protein levels in the culture supernatant after exposure to metals for 24 h were measured by ELISA. Data are mean ± SE of 3-4 individual cultures. $^{*}p<0.05$ versus 0 μM.

Figure 1

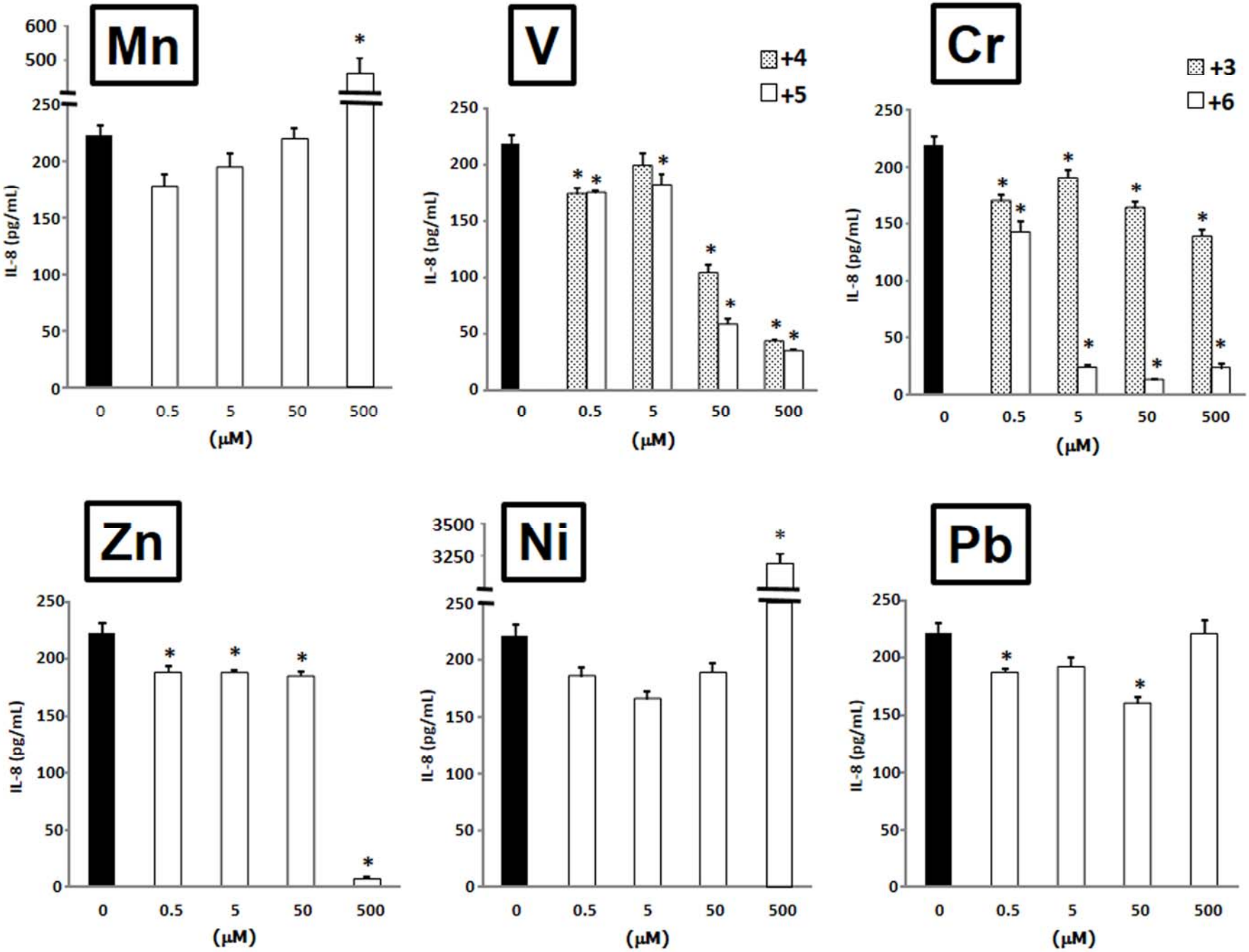

Figure 2

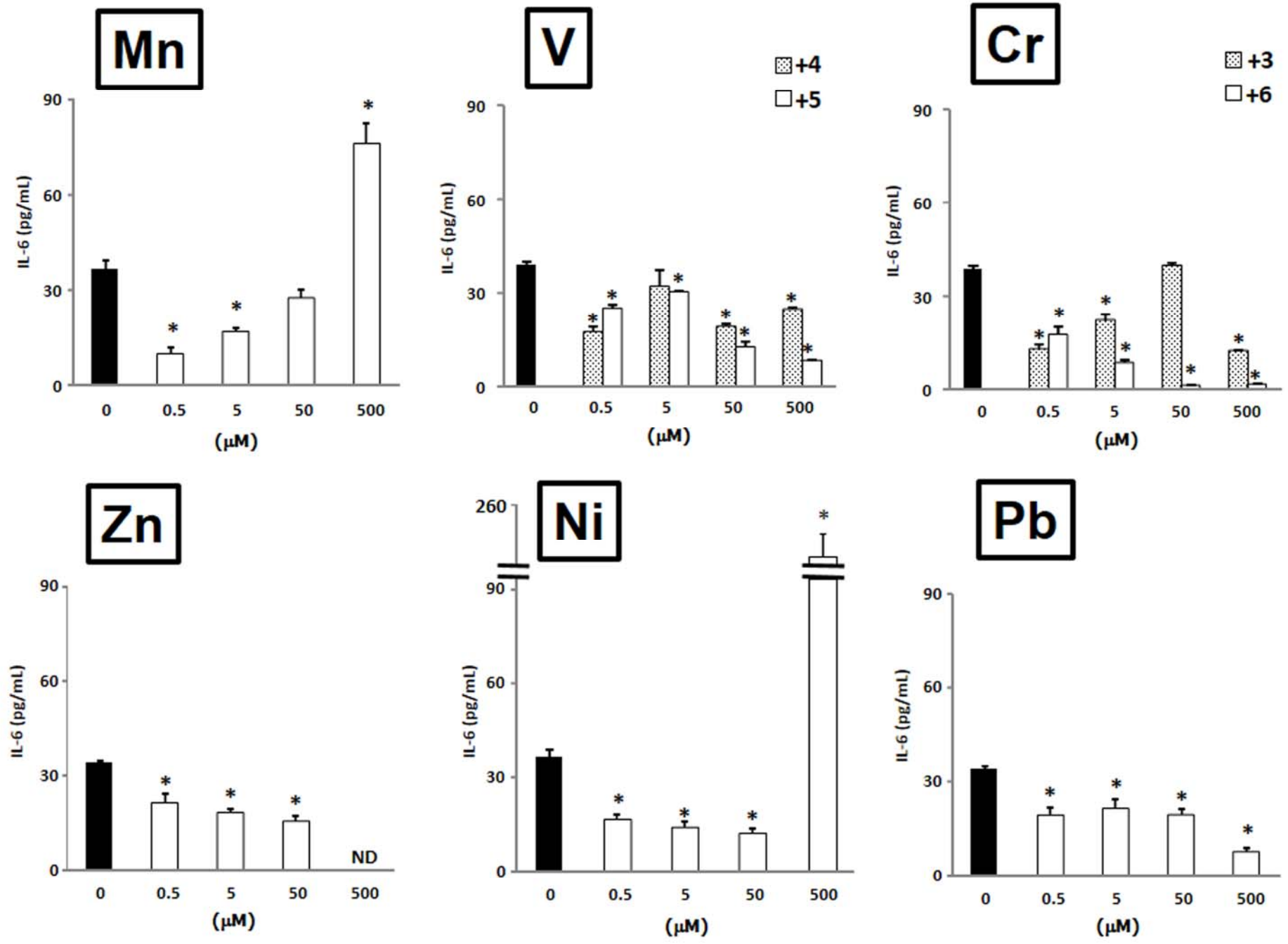

Figure 3