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Soluble transition metals cause the pro-inflammatory effects of welding fumes in vitro

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Abstract

Epidemiological studies have consistently reported a higher incidence of respiratory illnesses such as bronchitis, metal fume fever (MFF), and chronic pneumonitis among welders exposed to high concentrations of metal-enriched welding fumes. Here, we studied the molecular toxicology of three different metal-rich welding fumes: NIMROD 182, NIMROD c276, and COBTEL 6. Fume toxicity in vitro was determined by exposing human type II alveolar epithelial cell line (A549) to whole welding fume, a soluble extract of fume or the "washed" particulate. All whole fumes were significantly toxic to A549 cells at doses $>63 \mu\text{g ml}^{-1}$ (TD 50; 42, 25, and 12 $\mu\text{g ml}^{-1}$, respectively). NIMROD c276 and COBTEL 6 fumes increased levels of IL-8 mRNA and protein at 6 h and protein at 24 h, as did the soluble fraction alone, whereas metal chelation of the soluble fraction using chelex beads attenuated the effect. The soluble fraction of all three fumes caused a rapid depletion in intracellular glutathione following 2-h exposure with a rebound increase by 24 h. In addition, both nickel based fumes, NIMROD 182 and NIMROD c276, induced significant reactive oxygen species (ROS) production in A549 cells after 2 h as determined by DCFH fluorescence. ICP analysis confirmed that transition metal concentrations were similar in the whole and soluble fractions of each fume (dominated by Cr), but significantly less in both the washed particles and chelated fractions. These results support the hypothesis that the enhanced pro-inflammatory responses of welding fume particulates are mediated by soluble transition metal components via an oxidative stress mechanism.

Keywords: Welding fumes; Transition metals; Inflammation; Oxidative stress

Introduction

At present, it is estimated that more than one million workers are employed as welders worldwide (Sundin, 1998), with more than three million performing welding intermittently as part of their work duties (Sferlazza and Beckett, 1991). Manual metal arc welding (MMAW) and flux core arc welding (FCAW) are two commonly used procedures in the construction industry and in numerous other industrial processes. Welding involves the fusion of metals by high temperature generated via an electrical arc resulting in the formation of metal-enriched fumes. Welding fumes are a complex mixture of gases and small particulates of metal oxides formed by the vaporization

and oxidation of metal during the welding process (Lockey et al., 1988 and Yu et al., 2000). The nature of respirable fumes depends upon the type of welding and the composition of electrode, filler wire, and fluxes (Antonini et al., 1996; Lockey et al., 1988; Sferlazza and Beckett, 1991 and Yu et al., 2000).

Numerous studies have reported welding fume particles to be well within the respirable size range $<1 \mu\text{m}$ (reviewed by Antonini et al., 1998; Lockey et al., 1988 and Yu et al., 2000). Thus, upon inhalation, particle deposition occurs in the lower respiratory tract, including the terminal bronchioles and alveoli, beyond the mucociliary escalator (Sferlazza and Beckett, 1991 and Yu et al., 2000).

The health of welders has been studied extensively (Doig and Duguid, 1951 and Sferlazza and Beckett, 1991). A number of epidemiological studies have reported a higher incidence of respiratory illness such as bronchitis, airway irritation, metal fume fever (MFF), chemical pneumonitis, and also changes in lung function in welders (Antonini et al., 1996 and Sferlazza and Beckett, 1991). Furthermore, the severity, duration, and frequency of acute upper and lower respiratory tract infections have been shown to be greater among welders compared to the general population (Howden et al., 1988).

The most frequently described respiratory illness among welders is metal fume fever (MFF), which is an acute, self-limiting, systemic, febrile illness caused by inhalation of high concentrations of metal oxides, primarily zinc oxide (Blanc et al., 1993 and Sferlazza and Beckett, 1991). Symptoms include high fever and sweating, throat irritation, chest tightness, dry cough, and general malaise associated with a pulmonary inflammatory cellular response (Kuschner et al., 1995 and Lockey et al., 1988), peaking 5–12 h postexposure and resolving within 24–48 h (Antonini et al., 1996 and Sferlazza and Beckett, 1991). Although the pathogenesis of MFF is poorly understood, allergic and immunological mechanisms are most frequently postulated (Graeme and Pollack, 1998). Cytokine networking mediated by the release of pro-inflammatory cytokines TNF- α and IL-8 by pulmonary macrophage causes both local pulmonary inflammatory cellular response and systemic response (Blanc et al., 1993; Gordon, 1991 and Kuschner et al., 1995). This theory is consistent with evidence showing that tolerance to metal fumes develops and symptoms are not observed on successive days of fume exposure (Blount, 1990; Graeme and Pollack, 1998 and Nemery, 1990).

Reports of a dose-dependent increase in pro-inflammatory cytokines and neutrophilia in the human bronchoalveolar lavage (BAL) 20–22 h following zinc oxide inhalation provide further evidence supporting the role of a cytokine-mediated mechanism causing the symptoms of MFF (Blanc et al., 1993 and Kuschner et al., 1995). Furthermore, TNF- α levels were significantly greater 3 h postexposure, suggesting an initial role for TNF- α in the pathophysiology of MFF (Blanc et al., 1993 and Kuschner et al., 1997). Similar finding were reported in rats following intratracheal installation of stainless steel welding fumes with increased levels of TNF- α and IL- β in BALF (Antonini et al., 1996). These cytokines, produced predominantly by alveolar macrophages, are involved in numerous inflammatory processes such as neutrophil recruitment and increased oxygen radical production (Driscoll et al., 1990; Driscoll et al., 1991; Goldring and Krane, 1986; Schmidt et al., 1982 and Tsujimoto et al., 1986). These findings provide further evidence supporting the role of cytokines in

the inflammatory response associated with exposure to certain environmental and occupational particles (Antonini et al., 1996).

The importance of metal content, metal bioavailability, and interactions among transition metals in mediating pulmonary inflammation and injury following exposure to many different particles has been demonstrated. Such particles include residual oil fly ash (ROFA), stone quarry particles (Hetland et al., 2001), Provo PM10 (Costa and Dreher, 1997), and ambient airborne particles (Costa and Dreher, 1997; Dreher et al., 1997 and Gilmour et al., 1996). Furthermore, the pathogenicity associated with exposure to crocidolite-asbestos has been linked to the high iron content on fiber surfaces (Jiménez et al., 2000 and Weitzman and Weitberg, 1985).

Mechanistic studies report that particle-associated transition metals can undergo redox cycling resulting in the production and release of reactive oxygen species (ROS). These reactive compounds can deplete antioxidants, cause cellular damage, lung injury, and inflammation (Carter et al., 1997; Dreher et al., 1997; Jiménez et al., 2000; Stohs and Bagchi, 1995 and Toyokuni, 1996).

The aims of this study were to investigate the molecular toxicology of three compositionally different welding fumes by comparing their potential to activate lung epithelial cells to release the pro-inflammatory cytokine IL-8. We hypothesized that transition metals present in or on welding fume particles cause pulmonary inflammation and lung injury observed in welders. In addition, we postulated that the soluble metal components were the main contributing factors.

To investigate this we initially performed experiments to determine the toxicity of three different welding fumes on alveolar epithelial cells. To examine the possible mechanisms by which nontoxic concentrations of welding fume particles may cause lung inflammation, the ability of fumes to cause expression of the pro-inflammatory cytokine IL-8 in epithelial cells was assessed. A recent study by Antonini et al., 1999 identified the soluble components of fume from stainless steel MMAW to be most cytotoxic and have the greatest effect on macrophage function. Similar studies using other environmental particulates have shown that the soluble metal fraction plays an important role in the induction of lung injury (Dreher et al., 1997 and Adamson et al., 1999). Therefore, to determine which components of welding fume particles were responsible for the pro-inflammatory response, alveolar epithelial cells were exposed to soluble, insoluble, or whole fume components at subtoxic concentrations. In addition, we investigated whether welding fumes exerted some of their effects via oxidative stress by assessing the production of intracellular ROS and levels of the important antioxidant glutathione.

Materials and methods

Materials

All reagents were obtained from Sigma-Aldrich, UK unless otherwise stated.

Cell culture

The type II human alveolar-like epithelial cell line A549 (European Collection for Animal Cell Culture) was maintained in continuous culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine

serum (FBS), 2 mM glutamate, and 100 IU ml penicillin–1·100 µg ml^{−1} streptomycin–1. Cells were grown to confluence at 37 °C in a humidified atmosphere containing 5% CO₂, washed with Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS-CMF), harvested with Trypsin-EDTA, and passaged.

The cells were seeded at a density of 0.2 × 10⁶, 1.1 × 10⁶, or 9 × 10⁴ ml^{−1} in 6-, 24-, or 96-well culture plates, respectively, and grown until approximately 80% confluent in complete media. Cells were then washed with PBS-CMF and grown to confluence in media containing 2% FBS.

Welding fume particles

Samples of welding fume particles produced by two nickel-based stainless steel welding consumables ((NIMROD 182, and NIMROD c276) and one cobalt-based stainless steel welding consumable (COBTEL 6) were obtained from Health and Safety Laboratory, Sheffield, UK. These samples were generated under controlled conditions at The Welding Institute (TWI), Great Abington, Cambridge, UK, by carrying out mechanical welding inside a fume box and collecting the fume on paper filters. The resulting material was removed from the filters by brushing and stored in airtight glass containers until required.

Welding fume characterization

Welding fume particles were characterized as described by Brown et al. (2001). Briefly, fume particles were suspended at a stock concentration of 1 mg ml^{−1} in deionized water. Five microliters of each suspension was applied to the surface of 200-mesh size carbon-coated electron microscope (EM) grids (Agar Scientific). Grids were placed on filter paper and dried at room temperature and subsequently examined by transition electron microscopy (TEM).

Treatment of A549 cells with welding fumes

Welding fume particles were prepared at a stock concentration of 1 mg ml^{−1} in media containing 2% FBS (2% DMEM), sonicated for 10 min, and vortexed briefly to ensure complete dispersion. For all experiments after the initial dose-response assessments, monolayers were treated with concentrations ranging from 2 to 63 µg ml^{−1}. Cells were exposed to either whole particle suspensions, soluble fraction, or washed particles only at a concentration of 63 µg ml^{−1}. Nontoxic concentrations of TNF-α (10 ng ml^{−1}) (R&D systems, Abingdon UK) and carbon black (Huber 900) (100 µg/ml) (Degussa, Frankfurt, Germany) were used as positive control and an inert particle, respectively.

Soluble components were isolated from whole particle suspensions at the relevant concentration by centrifugation at 13 000 rpm (5 min) to pellet any particulates. The resultant supernatant was transferred into a clean Eppendorf tube and the procedure was repeated four times to ensure that any particulate contaminant was removed before treatment. The concentration of the soluble fraction was taken to be the concentration of the soluble components derived from the whole welding fume particle suspension at the indicated concentration.

To determine whether insoluble fume particulates had any effect on A549 cells, particulates were pelleted by centrifugation and repeatedly washed and centrifuged in

PBS-CMF. The resultant particles were resuspended in 2% DMEM and applied to monolayers for the time indicated.

Chelation of welding fumes

Whole particle suspensions and soluble fractions of welding fume particles (63 µg ml⁻¹) were suspended in 2% DMEM containing 50 mg ml⁻¹ chelex beads and mixed on a rotating wheel for 4 h at room temperature. After incubation, samples were centrifuged at 13 000 rpm (5 min) to pellet the chelex beads. The resultant suspensions were applied to monolayers and incubated at 37 °C for 24 h.

Cytotoxicity

Cytotoxicity was assessed by lactate dehydrogenase (LDH) release. Cells seeded in 96-well flat bottomed culture plates were grown to confluence in media containing 2% FBS and exposed to a range of fume particle concentrations (1–250 µg ml⁻¹) for the various times indicated. LDH release was measured according to manufacturers instructions (Roche Molecular Biochemicals) using pyruvic acid as a substrate. LDH activity was determined spectrophotometrically at 490 nm and expressed as percentage of total cellular LDH, which was measured in the cell lysates obtained by treatment with 0.1% Triton X-100.

Assessment of metabolic activity

The ability of A549 cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2; diphenyl tetrazolium bromide (MTT) was used as an indicator of the metabolic competence of the cells and was performed as described by Mosmann (1983) with minor modifications. Cells were seeded at a density of 0.1 × 10⁶ cells ml⁻¹ in 24-well flat-bottomed plates and grown until approximately 80% confluence in media containing 10% FBS. Cells were washed with PBS-CMF and grown until confluent in 2% DMEM. Monolayers were treated with particle suspensions in concentrations of 1–250 µg ml⁻¹ for 4–48 h. Four hours before harvest, 100 µl MTT (0.5 mg ml⁻¹) was added and incubated for a further 4 h. Following incubation, isopropanol/0.1 M HCl (500 µl) was added to each well, mixed thoroughly, and centrifuged at 2000 rpm for 5 min to sediment any particles. Supernatants (200 µl) were transferred in triplicate to a flat-bottomed 96-well plate and the absorbance was determined spectrophotometrically on a microplate reader at 570 nm (reference wavelength: 630 nm). MTT reduction for each treatment was expressed as a percentage of control values.

IL-8 mRNA by RT-PCR

Following treatment, cells were washed with PBS-CMF and total cellular RNA was isolated using Trizol Reagent (GIBCO-BRL) according to manufacturers instructions and dissolved in 50 µl diethylpyrocarbonate (DEPC)-treated water. RNA (2 µg) was reverse-transcribed using M-MLV RT (Promega) and the resultant cDNA was stored at -20 °C until required.

PCR was performed for the genes IL-8 and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 5 and 3 µl cDNA, respectively. Oligonucleotide primers used for PCR reactions were obtained from (MWG-Biotech AG).

Primer sequences were IL-8: sense 5'-ATT GAG AGT GGA CCA CAC TGC GCC-3', antisense 5'-CAC TGA TTC TTG GAT ACC ACA GAG-3'; GAPDH: sense 5'-

CCA CCA TGG CAA ATT CCA TGG CA-3', antisense 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'.

Conditions for PCR were IL-8: 31 thermal cycles of denaturation (94 °C for 1 min), annealing (60 °C for 1 min), extension (72 °C for 1 min), final extension for 5 min at 72 °C; GAPDH: 20 cycles of denaturation (72 °C for 90 s), annealing (94 °C for 30 s), extension (60 °C for 45 s), final extension for 10 min at 72 °C.

The amplification products were electrophoresed through a 1.5% agarose gel containing ethidium bromide. The resultant bands were scanned and visualized using a UVP High Performance Ultraviolet Transiluminator and GRAB-IT software version 2.5. The intensity of the IL-8 (173 bp) and GAPDH bands (600 bp) was determined by densitometry using GelBase/GelBlot software and expressed as a ratio of the band intensity of IL-8/GAPDH.

Enzyme-linked immunosorbent assay for IL-8

The IL-8 protein was determined using monoclonal and biotinylated antihuman IL-8 antibodies obtained from NIBSC (Pottersbar, Hertfordshire, UK) for initial dose-course experiments or R&D Systems (Oxon, UK) for all subsequent ELISA. Briefly, 96-well microplates (EIA/RIA, Costar, Cambridge) were coated with monoclonal IL-8 antibody (2 µg ml⁻¹, NIBSC; or 4 µg ml⁻¹, R&D). Cytokine levels of test supernatants were assessed according to manufacturers instructions utilizing a standard quantitative immunometric "sandwich" enzyme technique, with values being determined using a recombinant protein standard curve ranging from 5 to 800 pg ml⁻¹ IL-8. All samples were analyzed in triplicate and IL-8 protein levels were expressed as a percentage of the control.

Total glutathione reduced (GSH) + oxidized (GSSG)

Following treatment with either whole particulate supernatant or soluble fractions only, for the time intervals indicated, cells were washed with PBS-CMF, harvested with trypsin-EDTA, and rewashed. Total intracellular glutathione was measured by the DTNB/GSSG reductase recycling method described by Tietze (1969) with slight modifications. Total GSH concentration was determined using linear regression of a GSH standard curve. The values were expressed as the amount of GSH in µmol per mg of protein as a percentage of the control.

Intracellular reactive oxygen species

The level of intracellular ROS was determined by the change in fluorescence resulting from the oxidation of the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). Cells exposed to either whole particle suspensions or soluble fractions only were incubated with 40 µM of the fluorescent probe DCFH-DA for 30 min at 37 °C. Following incubation, cells were washed twice with PBS-CMF, harvested by trypsinization (0.25% Trypsin-EDTA), rewashed, and resuspended in PBS-CMF. The degree of fluorescence, corresponding to intracellular ROS, was determined using FACS Calibular flow cytometry (excitation λ 488 nm; emission 530 nm). The proportion of fluorescence cells was determined using Cell Quest software and expressed as a percentage control geo-mean fluorescence.

Metal analysis

Samples of whole particle suspension, washed particle suspension, soluble fraction, and chelex-treated soluble fraction were prepared, as above, from an initial welding fume concentration of 63 $\mu\text{g ml}^{-1}$, as applied in the biological assays. One milliliter of each sample was diluted to 5 ml in ultrapure water and the aqueous fraction analyzed by ICP-MS (VG Elemental PlasmaQuad 3) for the transition metals Co, Cr, Cu, Fe, Mn, Ni, Ti, V, and Zn selected for their potential to take part in redox cycling. Sample concentrations were quantified against 11-point calibration graphs constructed using a multi-element ICP standard.

Statistical analysis

Individual experiments were conducted in triplicate unless otherwise stated. Data are expressed as means \pm SEM ($n = 3$) and were analyzed on MINITAB version 10.5. Statistical significance was determined using one-way analysis of variance (ANOVA) with post hoc Tukey's pairwise comparisons. Significance is reported at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control; \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ NIMROD 182 vs. NIMROD c275 or # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ NIMROD 182 vs. COBTEL 6.

Results

Welding fume characterization

TEM images of welding fume particles are shown in Fig. 1. All welding fumes were heterogeneous in nature containing a high proportion of ultrafine (uf) particles with some larger particles. Images of carbon black and ultrafine carbon black (Figs. 1D and E) are shown for comparison.

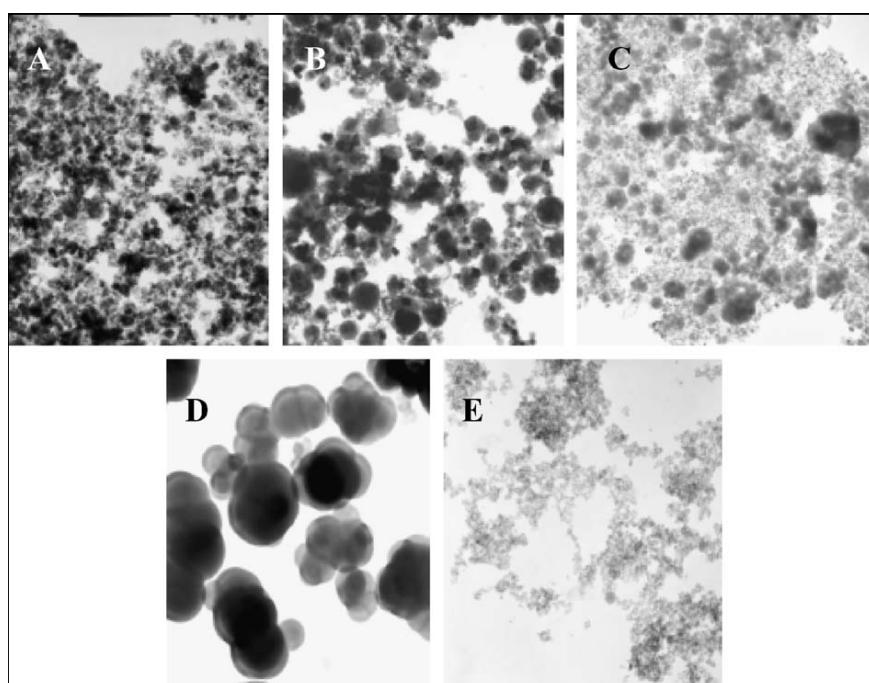


Fig. 1. Transition electron micrographs showing particles. (A) NIMROD 182. (B) NIMROD c276. (C) COBTEL 6. (D) Carbon Black. (E) Ultrafine Carbon Black. Magnification: $\times 35\,000$.

Cytotoxicity assessments

To establish the potential toxicity of the three welding fumes NIMROD 182, NIMROD c276, and COBTEL 6, alveolar epithelial cells were incubated with welding fumes (1–250 µg ml⁻¹) for 24 h and LDH release was measured in cell lysates. At concentrations greater than 63 µg ml⁻¹, all fumes caused significant toxicity ($P < 0.005$) (Fig. 2). Furthermore, following treatment, all three welding fumes caused a significant dose and time-dependent decrease in the metabolic activity of alveolar epithelial cells as measured by the reduction of MTT (Fig. 3). There was also a clear time-dependant depletion in MTT (data not shown).

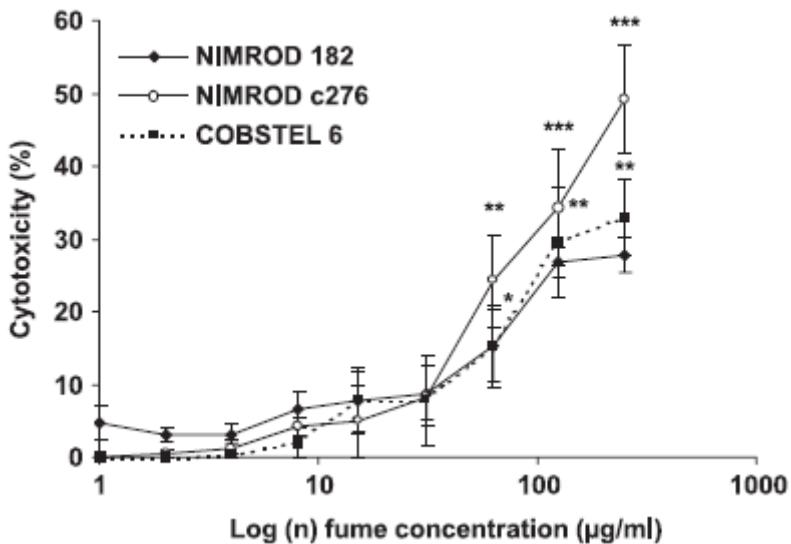


Fig. 2. Welding fume particles cause increased lactate dehydrogenase (LDH) release in alveolar epithelial cells. LDH release is expressed as percentage of total cellular LDH measured in the cell lysates obtained by treatment with 0.1% Triton X-100 following treatment with welding fumes (1–250 µg ml⁻¹) for 24 h. The graph represents the mean of three experiments conducted in triplicate and the bars represent \pm SEM * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ compared to 100% cytotoxicity from 0.1% Triton X-100.

NIMROD 182 was found to be less toxic than either NIMROD c276 or COBTEL 6 when compared to control values at lower concentrations. However, at the higher doses tested (63, 125, 250 µg ml⁻¹), there was no significant difference between fumes. As the experimental aim was to investigate the molecular mechanisms of the pro-inflammatory effects of welding fumes rather than their direct toxicity, all subsequent experiments were performed using nontoxic concentrations (2–63 µg ml⁻¹). Acellular experiments showed no interference with either assays from welding fume particles alone (data not shown).

The toxic dose causing 10%, 25%, or 50% toxicity was calculated. TD_x of each welding fume was calculated using results obtained from the above assays (Tables 1A and B).

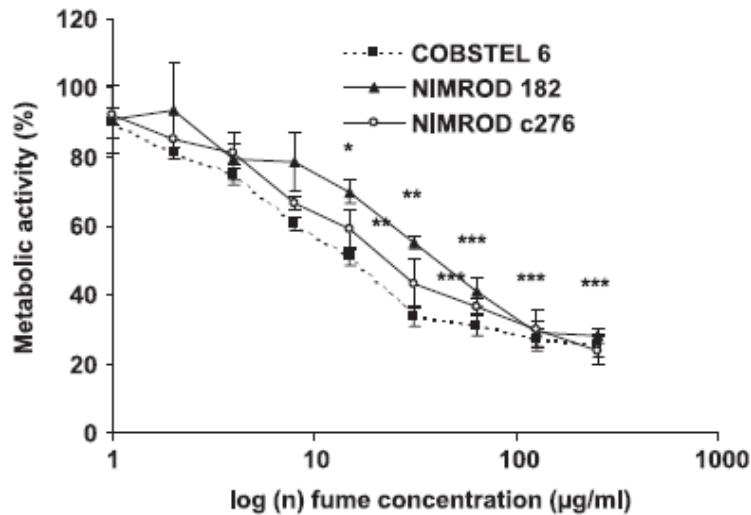


Fig. 3. Welding fume particles decrease the metabolic activity of alveolar epithelial cells. Metabolic activity was determined by the reduction of MTT following treatment with welding fumes (1–250 µg ml⁻¹) for 24 h and is expressed as percent of the control value (0 µg ml⁻¹). The graph represents the mean of four experiments conducted in triplicate. The bars represent \pm SEM *P < 0.05, **P < 0.005, ***P < 0.001 compared to control.

A. Toxic dose of welding fumes determined by LDH release

Sample	TD 10	TD 25
NIMROD 182	37.5	117.5
NIMROD c276	35	70
COBTEL 6	42.5	108.75

B. Toxic dose determined by reduction of metabolic activity of epithelial cells

Sample	TD 25	TD 50
NIMROD 182	11.2	42
NIMROD c276	6.25	25
COBTEL 6	4	11.875

IL-8 protein production

Enzyme-linked immunosorbance assay (ELISA) was used to assess IL-8 protein production from A549 cells exposed to welding fumes. A549 cells were treated with welding fumes at concentrations between 2 and 63 µg ml⁻¹ for 6 and 24 h. At higher concentrations (31 and 63 µg ml⁻¹), both NIMROD c276 and COBTEL 6 caused a significant increase in IL-8 production at both time points (P < 0.01, P < 0.001) (Figs. 4A and B). Following 6-h exposure, NIMROD c276 induced the highest cytokine response in A549 cells. At both time points, NIMROD 182 was the least potent fume for IL-8 release. Following 6-h exposure to NIMROD 182 (63 µg ml⁻¹), a 4-fold increase in IL-8 release was measured in comparison to the 13-fold and 8-fold increase induced by NIMROD c276 and COBTEL 6, respectively (Fig. 4a).

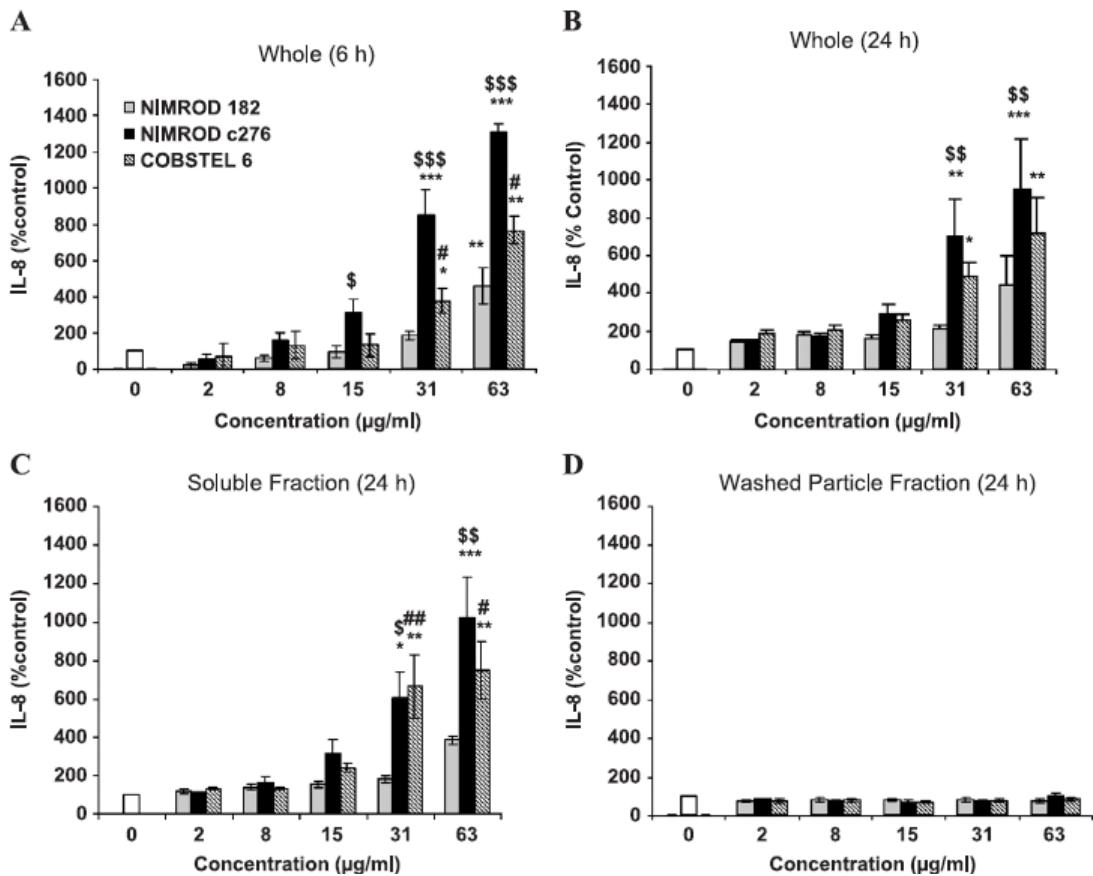


Fig. 4. Effect of welding fume particles on IL-8 protein. A549 cells were treated with welding fumes ($2-63 \mu\text{g ml}^{-1}$) for (A) 6 or (B) 24 h. (C) Cells were exposed to the soluble fraction of welding fumes. (D) Welding fume particles were washed for 24 h and the supernatant was analyzed for IL-8 protein. The values are expressed as percent control ($0 \mu\text{g ml}^{-1}$). The histograms represent the mean of four experiments performed in triplicate and the bars represent SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control. \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.005$ NIMROD 182 v NIMROD c276, # $P < 0.05$, ## $P < 0.01$ NIMROD 182 v COBTEL 6.

We hypothesized that the soluble metal components of welding fumes play a key role in the pro-inflammatory effects. To address this issue, alveolar epithelial cells were treated with either the soluble components or washed particles and IL-8 release was measured following 24-h exposure. The soluble fraction from all three fumes enhanced IL-8 expression at the highest concentrations (31 and $63 \mu\text{g ml}^{-1}$). The levels of IL-8 measured were equivalent to those observed following exposure to whole fume suspensions (Figs. 4C and B). No increase in IL-8 production was shown after 24-h treatment with washed fume particles (Fig. 4D). These findings suggest that the soluble components are responsible for all of the pro-inflammatory effects of welding fumes.

To investigate the time-scale over which soluble components exert their inflammatogenic effects, the particles were suspended in supernatant over a 24-h period. At various time points, the supernatant was collected, centrifuged to remove any particles, and alveolar epithelial cells were treated with them for 24 h and IL-8 release was measured.

Supernatant obtained following 10-min incubation with all three fumes significantly enhanced IL-8 expression in A549 cells (Table 2). At all other time points, IL-8 production was similar to that measured at the initial time point showing that all of this soluble activity was released in the first few minutes.

Fume	Time (h)						
	0	0.16	0.5	1	4	8	24
NIMROD 182	50.24 ± 5.2	258.44 ± 15.9***	221.67 ± 5.4***	191.80 ± 10.3***	216.62 ± 15.9***	227.65 ± 23.1***	210.64 ± 12.5***
NIMROD c276	50.24 ± 5.2	291.53 ± 7.4***	265.34 ± 6.5***	285.56 ± 7.1***	310.84 ± 4.9***	294.75 ± 7.1***	337.95 ± 5.6***
COBTEL 6	50.24 ± 5.2	231.78 ± 6.1***	188.12 ± 14.0***	215.24 ± 12.1***	212.48 ± 20.1***	195.02 ± 8.3***	200.07 ± 6.7***

Table 2. Time course of release of soluble fractions of welding fume particles

Welding fume particles (63 µg ml⁻¹) were agitated over a 24-h period and supernatant was collected after 0.16, 0.5, 1, 2, 4, 8, and 24 h. Soluble fractions were isolated as explained in Materials and methods. A549 cells were then treated with the soluble fractions for 24 h and the supernatant was analyzed for IL-8 protein. The values are expressed as picograms per ml ± SEM.

IL-8 gene expression

RT-PCR analysis was used to evaluate changes in steady-state gene expression of IL-8 in A549 cells exposed to either whole welding fumes or soluble fraction alone (63 µg ml⁻¹) for 6 or 24 h. All three fumes significantly increased IL-8 mRNA at the 6 h time point (Fig. 5C). More importantly, there was no difference between the whole particle suspensions; that is, particles plus any soluble components, compared to the soluble fraction alone. By 24 h, mRNA expression had returned to basal levels (data not shown), suggesting that welding fumes elicit their inflammogenic response within the first few hours of exposure.

Chelation of soluble welding fumes

To assess the contribution of soluble transition metal components in inducing cytokine release, welding fumes were treated with the transition metal chelator, chelex beads (50 mg ml⁻¹) before addition to epithelial cells. We expected that transition metals released or mobilized from the fumes would be chelated by the beads and removed from the solution following centrifugation and removal of the beads. A significant reduction in IL-8 protein release was observed following treatment of supernatants of all three welding fumes with chelex beads when compared to untreated supernatant (Fig. 6). These findings provide further evidence for the role of soluble transition metals in the induction of the pro-inflammatory effects of welding fumes.

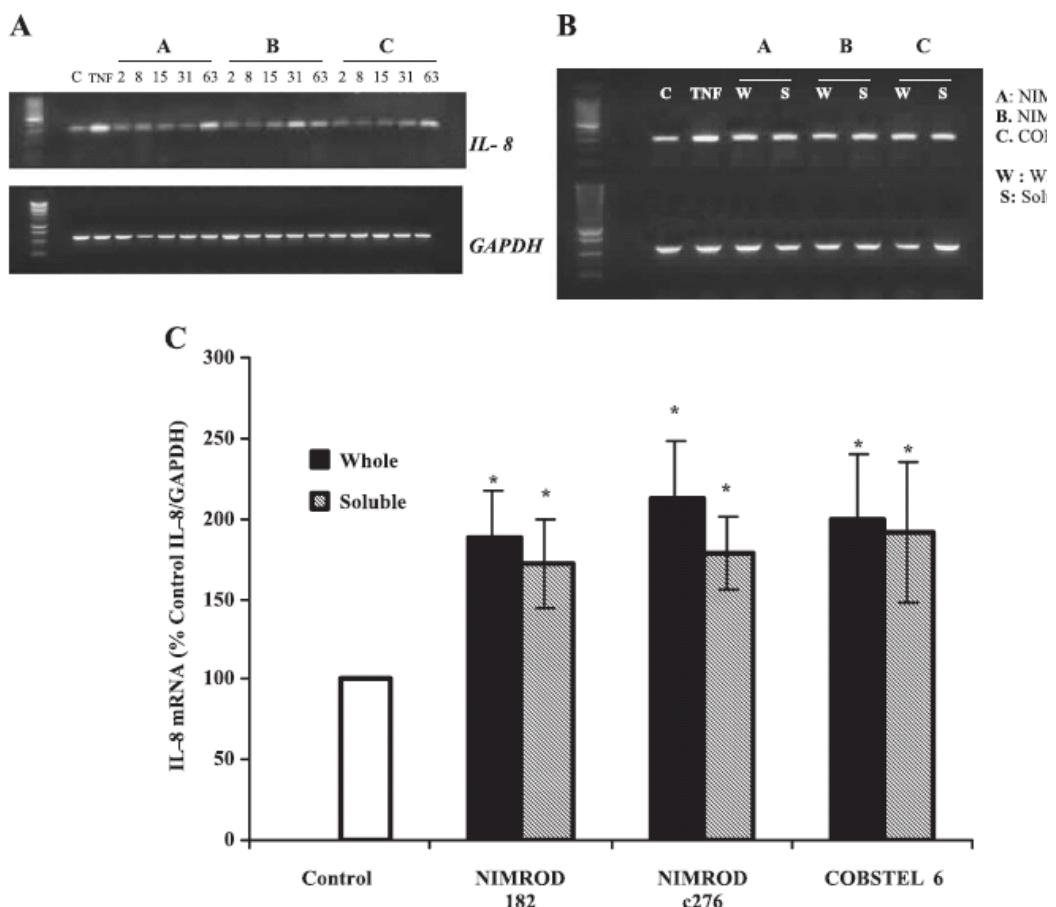


Fig. 5. Effect of welding fume particles on IL-8 gene expression. A549 cells were treated with welding fume particles 2–63 $\mu\text{g ml}^{-1}$ for 6 h, RNA was isolated, and IL-8 mRNA was quantified by RT-PCR. (A) Representative PCR gel for IL-8 and GAPDH for dose-response of A549 cells treated with welding fumes. (B) Representative PCR gel for IL-8 and GAPDH for cells exposed to either whole or soluble fraction of welding fumes. (C) Histogram represents $n = 4$ conducted on pooled triplicate samples \pm SEM. Values expressed as the ratio of IL-8 mRNA/GAPDH expressed as percentage control (0 $\mu\text{g ml}^{-1}$).

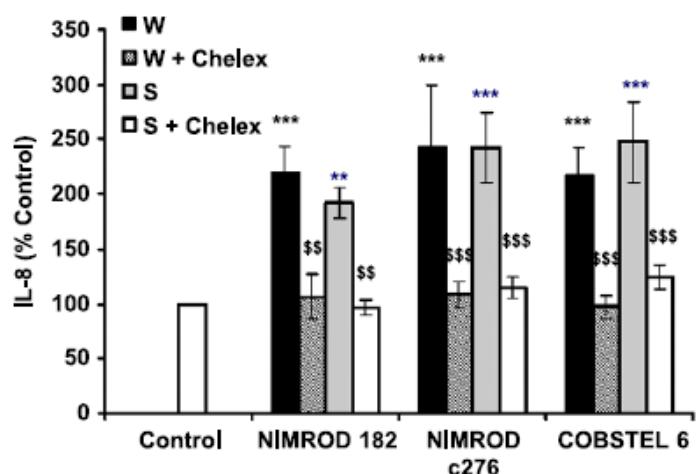


Fig. 6. Transition metal chelation inhibits cytokine release induced by welding fume particles and soluble fractions. Whole welding fume particles (W) (Black) and soluble

fractions (S) (Grey) ($63 \mu\text{g ml}^{-1}$) were treated with chelex beads (50 mg ml^{-1}) for 4 h (whole welding fumes + beads Black dashed), (soluble fractions + beads Grey dashed). A549 cells were then treated with either pre- or untreated fumes for 24 h and the supernatant was analyzed for IL-8 protein. The values are expressed as percent control ($0 \mu\text{g ml}^{-1}$). The histograms represent the mean of three experiments performed in triplicate and the bars represent the SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control. \$\$P < 0.01, \$\$\$P < 0.001 untreated welding fumes.

Intracellular glutathione concentrations

To investigate the involvement of ROS in the inflammatory and toxic responses following particle exposure, levels of the intracellular antioxidant glutathione were measured as an indicator of oxidative stress. Treatment of alveolar epithelial cells with all three welding fumes ($63 \mu\text{g ml}^{-1}$) for 2 h caused a significant depletion in intracellular glutathione concentrations ($P < 0.05$) (Fig. 7). However, when exposure was extended over a 24-h period, glutathione concentrations increased above control levels, suggesting a rebound effect.

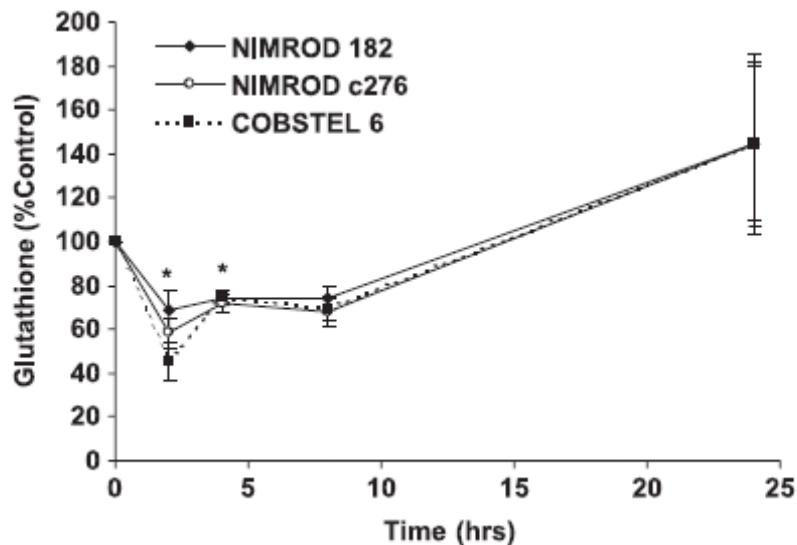


Fig. 7. Effect of welding fume particles on glutathione concentrations in alveolar epithelial cells. Total intracellular GSH concentration of A549 cells following treatment with whole welding fumes ($63 \mu\text{g ml}^{-1}$) for 0, 2, 4, 6, or 24 h. The values are expressed as glutathione percent control ($0 \mu\text{g ml}^{-1}$) at 0 h. The graph represents the mean of five experiments conducted in triplicate and the bars the SEM, * $P < 0.05$ compared to control.

To examine the mechanisms by which soluble components exert their effects, we compared total glutathione concentrations following 2-h exposure to either whole particle suspension or soluble components. Both whole particle suspensions and soluble components of each fume caused a similar degree of depletion in total glutathione compared to control (Fig. 8). These data further confirm that the soluble components exert their effect via oxidative stress.

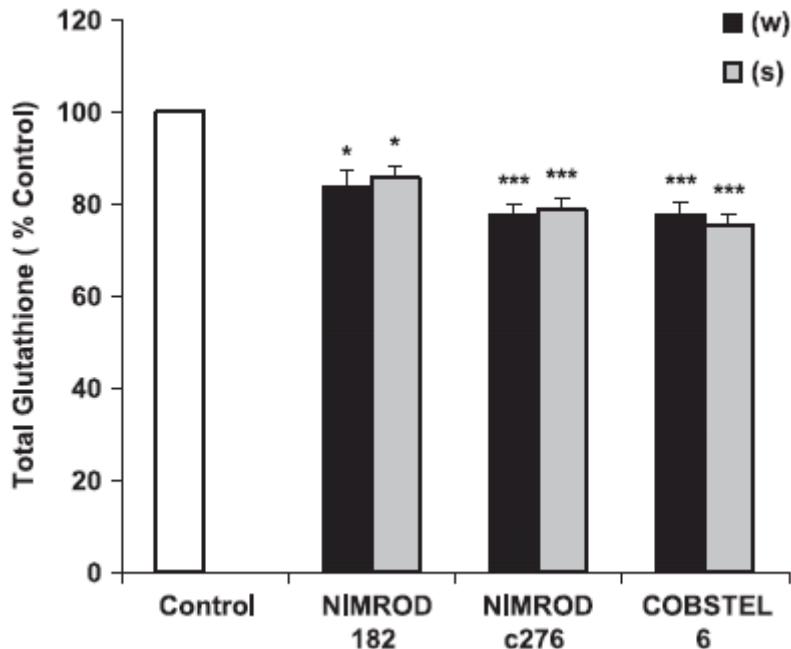


Fig. 8. Soluble fractions of welding fume particles deplete intracellular GSH. Total intracellular GSH following treatment with either whole welding fume particles (w) (black) or soluble fractions (s) (grey) ($63 \mu\text{g ml}^{-1}$) for 2 h. The values are expressed as percentage control. The graph represents the mean of five experiments conducted in triplicate and the bars the SEM, * $P < 0.05$, *** $P < 0.001$ compared to control.

Intracellular ROS levels

To further examine the possible mechanism by which welding fumes may cause an inflammatory response, we measured intracellular ROS levels in alveolar epithelial cells using the fluorescent probe DCFH-DA and flow cytometry. Both the soluble fraction and whole particle suspension of the nickel-based fumes, NIMROD 182 and NIMROD c276, caused a significant increase in intracellular ROS in epithelial cells following 2-h exposure ($P < 0.05$, $P < 0.005$) (Fig. 9). As both the soluble fraction and whole particle suspension caused a similar degree of oxidative stress, this further supports our hypothesis that the soluble components of welding fumes play a fundamental role in initiating the pro-inflammatory response.

However, the cobalt-based fumes, COBTEL 6, appeared to have no effect on the oxidative status of epithelial cells. This result is somewhat surprising as COBTEL 6 was shown to significantly deplete glutathione at the same time point (Fig. 8), which would suggest a role for ROS.

Metals analysis

The total concentration of all measured metals present in solution in each of the whole and washed particle samples, and soluble and chelex-treated fractions is shown in Fig. 10 for each welding fume. Fig. 10 clearly shows that the concentrations of metals were similar in the whole particle and soluble fractions, and were considerably greater than the concentration of metals in the washed particle fraction. The washed particle

fraction is synonymous with the particle core and is shown to differ with different welding fume particles (Fig. 10).

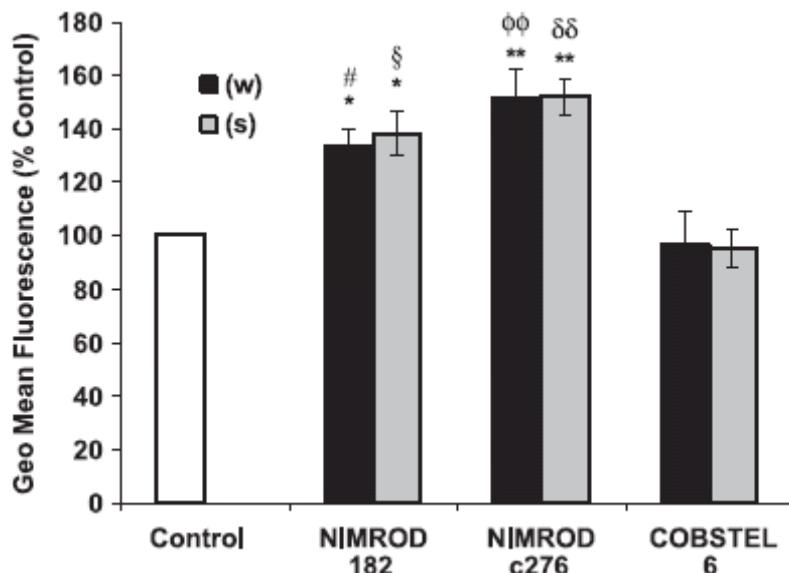


Fig. 9. Welding fume particles induce intracellular ROS production in alveolar epithelial cells. A549 cells were treated with either the whole welding fume particles (w) (black) or soluble fractions (s) (grey) ($63 \mu\text{g ml}^{-1}$) for 2 h and then incubated with the probe DCFH-DA and levels of intracellular ROS were determined by flow cytometry. Values are expressed as percent control ($0 \mu\text{g ml}^{-1}$) geo-mean fluorescence. Each graph represents the mean of six experiments conducted in triplicate and the bars represent the SEM, * $P < 0.05$, ** $P < 0.01$ compared to control. # $P < 0.05$, NIMROD 182 (w) vs. COBTEL 6 (w). § $P < 0.05$, NIMROD 182 (s) vs. COBTEL 6 (s); $P < 0.01$ NIMROD 182 (s) vs. COBTEL 6 (s). δδ $P < 0.01$ NIMROD 182 (s) vs. COBTEL 6 (s).

The chelex-treated samples also have low concentrations of transition metals. This pattern mimics very closely the observations of the inflammation in the bioassays and provides strong evidence that the soluble transition metal content of the welding fumes mediates the inflammatory responses.

For all three fumes, the soluble metal content was dominated by Cr (>80%) (of the metals measured). The COBTEL 6 fume also contained significant amounts of soluble Co. The remaining soluble metal content was composed principally of Mn, Ni, and Fe, with some Ti and V in the NIMROD c276 fumes (Fig. 10).

Discussion

The purpose of this study was to investigate the specific role of particle-associated transition metals in welding fumes in initiating pro-inflammatory effects in alveolar epithelial cells *in vitro*, to determine the importance of soluble metal in the welding fumes' ability to cause inflammation.

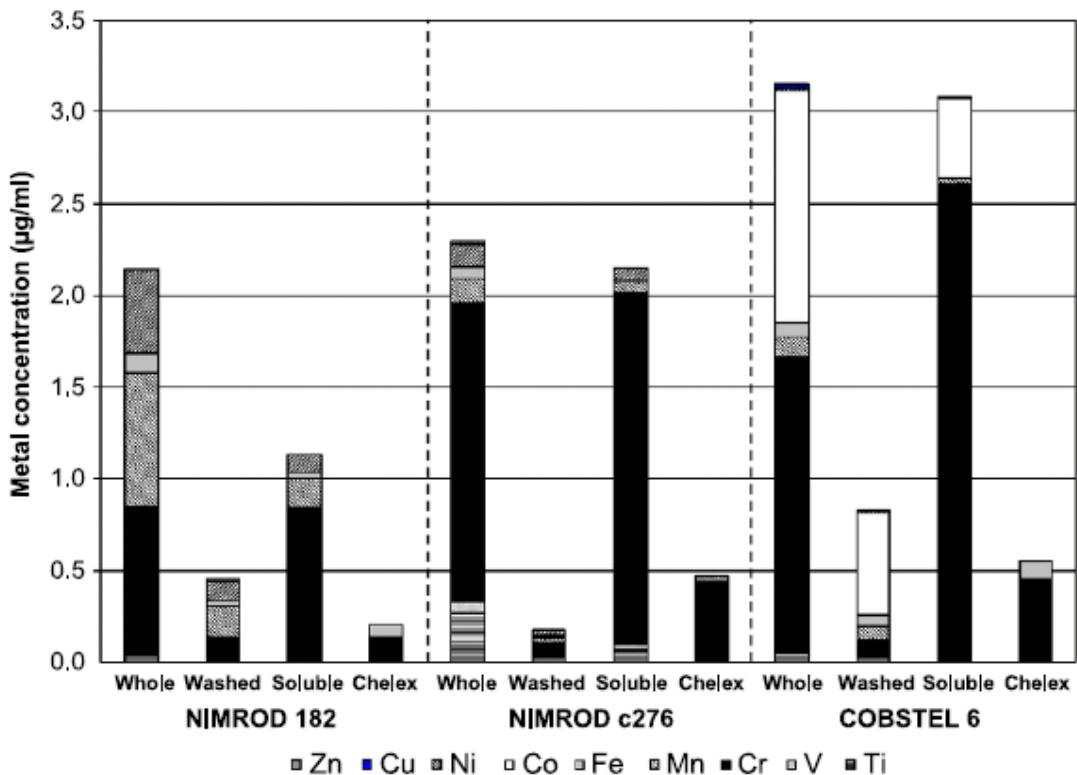


Fig. 10. Metal content of whole, soluble, washed, and chelex-treated soluble welding fume fractions. Transition metal content of welding fumes ($63 \mu\text{g ml}^{-1}$) was analyzed by ICP-MS and quantified using 11-point calibration from multi-element ICP standards. Values expressed as $\mu\text{g ml}^{-1}$ total metal. Total metal mass represents 5–6% of particle weight.

All of the fumes were cytotoxic at high doses ($>63 \mu\text{g ml}^{-1}$); however, following inhalation exposure, the deposited mass is likely to be very low due to effective clearance mechanisms such as the mucociliary escalator and alveolar macrophages, consequently, cell stimulation is the most probable mechanism. Therefore, to investigate the role of cell stimulation, we only utilized nonlethal concentrations for studies on ability to stimulate IL-8 release.

We demonstrated that exposure of alveolar epithelial cells to the welding fumes, NIMROD c276 and COBTEL 6, induced a significant increase in the production of IL-8 with a more pronounced affect at 6 than 24 h. However, NIMROD 182 was the least potent fume, inducing only a 4-fold increase above control at 6 h compared to 13-fold and 8-fold increases following exposure to NIMROD c276 and COBTEL 6, respectively. We suggested that the difference in welding fume particle potency may be due to the lower total metal concentration present in this fume compared to both NIMROD c276 and COBTEL 6. Previous studies have shown that different fluxes and shielding gases used during the welding process may affect the oxidation state of the fumes generated (Eagar et al., 1997). Consequently, the pulmonary responses of welding fumes vary according to the materials and processes used (Antonini et al., 1996). As the welding fumes examined in this study were obtained from compositionally different fluxes, and different welding processes were employed, the composition of the resultant fumes varied slightly.

It is widely accepted that transition metals such as iron, copper, and chromium undergo redox cycling, while nickel and cadmium cause the production of ROS and consequent GSH depletion (Stohs and Bagchi, 1995). Recent studies have demonstrated that the pulmonary toxicity of complex metal-containing particulates can be associated with the soluble forms of transition metals and the dose (Adamson et al., 2000; Carter et al., 1997; Dreher et al., 1997; Hetland et al., 2001; Kennedy et al., 1998 and Kodavanti et al., 1997). Indeed, particles containing easily solubilized metals appear to cause a more rapid onset and severity of acute pulmonary injury (Dreher et al., 1997). Furthermore, a study by Antonini et al. (1998) demonstrated that the solubility of welding fumes influenced the viability and ROS production in lung macrophages *in vitro*. There is also considerable evidence supporting the role of ultrafine (uf) particles (<0.1- μ m diameter) in mediating such responses. Previous work has demonstrated that uf particles induce a greater inflammatory response and free radical activity than fine counterparts of the same material, and this enhanced reactivity could be attributed to the larger surface area (Brown et al., 2001; Ferin et al., 1992 and Li et al., 1999). Several studies have reported that fumes generated by welding are <0.5- μ m diameter and are therefore likely to be deposited in the bronchiolar and alveolar regions of the lungs (Aksellsson et al., 1976 and Howden et al., 1988). As all three welding fumes were composed predominantly of (uf) particles (data not shown), any soluble metals present on particle surfaces may generate more free radical activity and thus induce a greater inflammatory response. In this study, we attempted to elucidate the specific components of welding fumes responsible for inducing inflammation by exposing epithelial cells to the soluble fraction and the washed particle fractions of each fume. Our results demonstrated following treatment with the washed particles, IL-8 release remained at basal levels. However, by 6 h, the soluble components of all three fumes significantly increased IL-8 mRNA expression and subsequent protein release by 24 h. More importantly, these results mimicked those observed following exposure to the whole welding fumes. This supports our hypothesis that the soluble components of all three welding fumes studied were entirely responsible for the increased pro-inflammatory responses observed in epithelial cells, and in this instance particle size or surface area did not contribute to the inflammatory response as has been proposed for a range of low toxicity particles (Duffin et al., 2001).

Having clearly established that the soluble fractions play a fundamental role in initiating welding fume toxicity, we further examined the involvement of transition metals in cytokine release by treating the soluble fractions of welding fumes with the chelator, chelex-100. Chelex-100 works on the principle that any cation present in solution is chelated onto the surface of the beads and thus, any released or mobilized transition metals are removed from the soluble fraction (Gao et al., 2002). Although there is evidence that small amounts of nonmetal chemical constituents and organic materials such as DNA and amino acids can be removed by chelation, this only occurs in alkaline condition (Giraffa et al., 2000; Hemmasi and Bayer, 1975 and Molinelli et al., 2002). As treatments were all slightly acidic (data not shown), chelation treatment should only remove soluble transition metals. Following pretreatment with chelex-100, IL-8 expression was completely attenuated, returning to basal levels (Fig. 6). The observation that IL-8 production is attenuated by chelex treatment of the samples is entirely consistent with the data in Fig. 10, which show that chelex-treated samples contain very significantly less soluble transition metal than the whole particle and soluble fractions that were not chelex-treated. Fig. 6 and Fig. 10 together provide

clear evidence for the role of soluble transition metals in the induction of inflammatory gene expression by welding fumes. These data corroborate findings by Pritchard et al. (1996), Kadiiski et al. (1999), and Dye et al. (1999) who demonstrated an association between the soluble metal content and enhanced pulmonary responses for other particle types. Furthermore, as all three fumes readily released their metals within a few minutes *in vitro*, we predict that the same would occur *in vivo* in lung lining fluid, leading to the acute effects seen in MFF.

As we carried out these experiments in serum-containing media, it could be argued that differential effects of metal-binding proteins could occur. However, an animal study has been carried out that entirely supports the conclusions obtained here confirming that the toxicity of welding fume-derived metals is similar in the lung as in cells treated in media containing serum.

Transition metals associated with welding fumes may elicit their pro-inflammatory effects via the generation of ROS through the catalysis of Fenton-like reactions (Stohs and Bagchi, 1995). Alternately, they could act via mechanisms independent of their oxidant catalytic activity (Dye et al., 1999). Glutathione (GSH) is one of the major intracellular antioxidants playing a vital role in maintaining the intracellular redox status (Rahman, 1999; Rahman and MacNee, 1998 and Rahman and MacNee, 2000). Both ROFA and diesel exhaust particles have been reported to deplete GSH (Dye et al., 1999). Therefore, we measured the levels of GSH in cells treated with welding fumes as a marker of oxidative stress.

Our results demonstrated that all three welding fumes caused rapid depletion in intracellular glutathione following 2-h exposure with a rebound increase observed after 24 h. Further examination demonstrated that depletion in GSH could be attributed to the soluble fraction alone. GSH depletion at 2 h (Fig. 8) was markedly lower in Fig. 7 (80% depletion compared to 40–60% depletion) due to the higher level of basal GSH in control cells. These findings support previous studies which report a similar effect in GSH levels following exposure to oxidants (Rahman, 1999; Rahman and MacNee, 1998 and Rahman et al., 1996) and are consistent with the respiratory syndrome metal fume fever (MFF), characterized by an acute pulmonary inflammation (Blanc et al., 1993; Graeme and Pollack, 1998 and Sferlazza and Beckett, 1991), adding further support for the role of oxidative stress in initiating MFF.

Oxygen radicals and their metabolites (ROS) have been shown to play a major role in pulmonary toxicity caused by the inhalation of different particles and fumes (Vallyathan and Shi, 1997). *In vitro* studies using various cell lines have demonstrated the involvement of ROS in inflammatory and toxic responses following particle exposure (Dye et al., 1999 and Pritchard et al., 1996). Furthermore, depletion of antioxidants such as glutathione may also result in ROS production. Therefore, we attempted to establish a link between GSH depletion and intracellular ROS production following exposure to the soluble components of welding fumes and thus determine whether ROS was responsible for the differences in inflammogenicity between fumes. Alveolar epithelial cells exposed for 2 h to the soluble fractions of both nickel-based fumes, NIMROD 182 and NIMROD c276, caused a significant increase in intracellular ROS production. The rise in ROS production may occur due to direct redox cycling of transition metals or as an indirect effect of GSH depletion.

Although COBTEL 6 contains numerous transition metals known to undergo redox cycling, such as Fe, Mn, and Cr, the production of ROS as measured using the fluorescent probe DCFH-DA did not appear to play a role in the molecular toxicity of COBTEL 6, as no difference in DCF fluorescence was observed. This result is somewhat surprising as we previously reported that soluble components of COBTEL 6 caused depletion of GSH to the same extent as both NIMROD 182 and NIMROD c276, suggesting the involvement of intracellular ROS. Although previous studies have identified metal-induced ROS production using the DCFH-DA fluorescent probe, these concentrated on specific metal compounds, such as Ni, Cr, and Co (Huang et al., 1993; Martin et al., 1998 and Salnikow et al., 2000). We speculate that the lack of ROS detected in our system may be because the chemical properties of welding fumes are highly complex, and the interactions between different metals may synergize, attenuate, or even block free radical formation detectable by this system. Furthermore, it is difficult to oxidize cobalt to Co^{+3} from its normal Co^{+2} oxidation state, in contrast to the more facile oxidation of other transition metals. Consequently, the cobalt present in COBTEL 6 may not be as effective a redox cycler for the generation of specific ROS detected by DCFH-DA. This observation is consistent with those of Salnikow, who saw no detectable ROS in A549 cells treated with CoCl_2 at a concentration of 100 μM (50-fold higher than in our fume).

Conclusion

In conclusion, these studies demonstrate that the soluble fractions of welding fumes play a fundamental role in mediating pro-inflammatory responses in alveolar epithelial cells as shown by increased expression of IL-8. Further examination highlighted that the soluble metal component was entirely responsible for this effect. In addition, enhanced levels of ROS and concomitant depletion of the antioxidant GSH suggest an important role for metal-particulate mediated oxidative stress, although more detailed evaluation of the signaling mechanisms involved is required. These data support the hypotheses that an enhanced inflammatory response, mediated by transition metals and oxidative stress in the lungs following inhalation of welding fume particles, is a potential mechanism for MFF and potentially other adverse health effects of welding fume exposure.

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