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MULTIPLE PROTEIN KINASE PATHWAYS MEDIATE AMPLIFIED IL-6 RELEASE BY HUMAN LUNG FIBROBLASTS CO-EXPOSED TO NICKEL AND TLR-2 AGONIST, MALP-2

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Abstract

Microbial stimuli and atmospheric particulate matter (PM) interact to amplify the release of inflammatory and immune-modulating cytokines. The basis of this interaction, however, is not known. Cultured human lung fibroblasts (HLF) were used to determine whether various protein kinase pathways were involved in the release of IL-6 following combined exposure to the PM-derived metal, Ni, and *M. fermentans*-derived macrophage-activating lipopeptide 2 (MALP-2), a toll-like receptor 2 agonist. Synergistic release of IL-6 by MALP-2 and NiSO₄ was obvious after 8 h of co-stimulation and correlated with a late phase accumulation of IL-6 mRNA. Ni and MALP-2, alone or together, all lead to rapid and transient phosphorylations of ERK_{1/2} and JNK/SAPK of similar magnitude. p38 phosphorylation, however, was observed only after prolonged treatment of cells with both stimuli together. A constitutive level of PI3K-dependent Akt phosphorylation remained unchanged by Ni and/or MALP-2 exposure. IL-6 induced by Ni/MALP-2 co-exposure was partially dependent on activity of HIF-1 α and COX-2 as shown by targeted knockdown using siRNA. IL-6 release in response to Ni/MALP-2 was partially sensitive to pharmacological inhibition of ERK_{1/2}, p38, and PI3K signaling. The protein kinase inhibitors had minimal or no effects on Ni/MALP-2-induced accumulation of HIF-1 α protein, however, COX-2 expression and, more markedly PGE₂ production, were suppressed by LY294002, SB203580, and U0126. Thus, Ni/MALP-2 interactions involve multiple protein kinase pathways (ERK_{1/2}, p38, and PI3K) that modulate events downstream from the early accumulation of HIF-1 α to promote IL-6 gene expression directly or secondarily, through COX-2-derived autocrine products like PGE₂.

Keywords

airborne particulate-derived metals; mitogen-activated protein kinase; p38; phosphoinositide 3-kinase; cyclooxygenase; hypoxia-inducible factor-1 α ; innate immunity; cytokines

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest regarding the material appearing in this report.

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INTRODUCTION

Inhalational exposure to airborne particulate matter (PM), even at ambient levels, has been linked to multiple adverse respiratory and cardiovascular health consequences including premature death (Dockery *et al.*, 1993; Pope *et al.*, 2002; Englert, 2004). PM of smaller size (less than 2.5 μm) is considered especially hazardous, since a higher percentage of fine particulates are retained in the lung compared to larger particulates (Ferin *et al.*, 1992). Moreover, PM_{2.5} and “ultrafines” contain a high content of a variety of heavy metal toxins. Fugitive fly ash derived from the combustion of residual fuel oil (residual oil fly ash, ROFA) is widely used as a surrogate to study the biological effects on metal-containing PM (Ghio *et al.*, 2002).

It is clear from numerous animal and *in vitro* studies that the toxicity from ROFA, as well as other PM, involves initiation of inflammatory cascades within the lung. The precise cellular and molecular mechanisms regulating the changes in gene expression that underlie these toxic pulmonary effects, however, are still not completely understood (Bayram *et al.*, 1998; van Eeden *et al.*, 2001; Becker *et al.*, 2002; Becker *et al.*, 2005). While devoid of significant amounts of organic components, ROFA does contain relatively high quantities of transition metals, such as iron (Fe) and vanadium (V) mostly in the form of soluble sulfates, and these are often implicated in the acute toxicity of ROFA (Dreher *et al.*, 1995; Ghio *et al.*, 2002). Activation of multiple stress-induced signal transduction pathways and transcription factors, in part through metal-catalyzed oxidative stress, has been demonstrated in many studies (Quay *et al.*, 1998; Samet *et al.*, 1998; Samet *et al.*, 2002).

Numerous other metal species are also present in ROFA, although the relative amount of each metal varies widely depending on the source of the particles. Nickel (Ni) is frequently a major component of ROFA (Kodavanti *et al.*, 1998) and needs to be considered as a factor in the biological effects of ambient PM. Ni exposure in the lung is associated with immunological sensitization, epithelial dysplasia, asthma, fibrosis, and cancer (Benson *et al.*, 1987; Morgan and Usher, 1994; Bernstein, 1997; Oller *et al.*, 1997). More recently, elevated levels of Ni in ambient air have been linked to cardiovascular dysfunction (Lippmann *et al.*, 2006), increased hospitalizations (Bell *et al.*, 2009), and exacerbations of reactive airway disease (Patel *et al.*, 2009). We previously reported that both ROFA and nickel sulfate (NiSO₄) could interact with microbial stimuli, such as mycoplasma infection, to markedly amplify the release of the immunomodulatory cytokine, interleukin 6 (IL-6) by human lung fibroblasts (HLF) (Gao *et al.*, 2004). Macrophage activating lipopeptide-2 (MALP-2), a Toll-like receptor-2 (TLR-2) agonist derived from *Mycoplasma fermentans*, could recapitulate the effects of live infection suggesting an interaction (or a “cross-talk”) between TLR-2-dependent and Ni-dependent signaling processes. The specific mechanisms responsible for this interaction on IL-6 remain to be identified, although synergistic interactions between Ni and MALP-2 on release of IL-8 utilized hypoxia inducible factor 1 α (HIF-1 α)- and cyclooxygenase 2 (COX-2)-dependent signaling pathways (Brant and Fabisiak, 2008; Brant and Fabisiak, 2009).

Mitogen- and stress-activated protein kinase (MAPK/SAPK) pathways are signaling routes that play crucial roles in many aspects of inflammatory responses. Several MAPK pathways including p38, ERK_{1/2} (p42/p44) and c-Jun N-terminal protein kinase (JNK) are activated by microbial products (Takeda *et al.*, 2003), PM-derived metals (Samet *et al.*, 1998; Samet *et al.*, 2002), and Ni (Aiba *et al.*, 2003; Nemec and Barchowsky, 2009) and are implicated in the inflammatory responses. Phosphatidylinositol 3-kinase (PI3K) and its downstream partner, Akt, represents another signaling module with important effects on growth, survival, migration and cellular response to injury (Hawkins *et al.*, 2006; Rommel *et al.*, 2007). Furthermore, significant “cross-talk” between PI3K/Akt and other signaling cascades

including MAPK/SAPK is frequently observed (Ouchi *et al.*, 2004; Wu *et al.*, 2005). Hence, we sought to further define the molecular signaling mechanisms responsible for the synergistic interactions between Ni and microbial stimuli and hypothesized that specific MAPK and/or PI3K pathways play a role in the amplified production of IL-6 by HLF exposed to NiSO₄ and MALP-2.

METHODS

Materials

Cell culture medium and fetal bovine serum (FBS) were from Invitrogen (Gaithersburg, MD). Tissue culture plasticware was from Falcon (Becton-Dickinson, Franklin Lakes, NJ). Calf thymus DNA, Hoechst 33258 and NiSO₄•6H₂O were from Sigma (St. Louis, MO). Low endotoxin bovine serum albumin (BSA) was from Intergen (Purchase, NY) and *M. fermentans*-derived MALP-2 was from Alexis Biochemicals (San Diego, CA). Bradford protein assay reagent was from Bio-Rad (Hercules, CA). Murine leukemia reverse transcriptase, RNase inhibitor, deoxynucleotides, oligo-dT, and Taq DNA polymerase were from Promega (Madison, WI). Protein kinase inhibitors LY294002, SB203580, PD98059, U0126 and SP600125 were from ALEXIS Biochemicals. IL-6-specific enzyme-linked immunosorbent assays (ELISA) were performed using Duo-set antibody pairs obtained R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Cell culture and treatments

Human lung fibroblasts were isolated as outgrowths from explanted surplus biopsy tissues obtained during routine follow-up bronchoscopy of lung transplant recipients as previously described (Fabisiak *et al.*, 1993) in accordance with a protocol approved by the University of Pittsburgh IRB. Individual cell lines prepared from previously frozen stocks were propagated in Minimal Essential Medium (MEM) supplemented with fetal bovine serum (10%, final concentration), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) in a humidified incubator with 5% CO₂/95% air as previously described (Fabisiak *et al.*, 2006). All cultures were negative for mycoplasma as determined by fluorescent microscopy using Hoechst 33258 (Chen, 1977) or MycoAlert™ mycoplasma detection kit (Cambrex BioScience, Rockland, ME). Time course and order of addition experiments were performed using individual cell lines each derived from different single donors with replicates representing at least 5 different lines. In order to minimize donor-to-donor variability all other experiments were performed using composite cell lines prepared from pooling early passage cells from 5 – 6 donors and repeated at least 3 times using these pooled cell preparations. For experiments, HLF were seeded into either 24-well plates (7 × 10⁴ cells/well/1 ml) for drug treatment effects on IL-6 release or 6-well plates (4 – 6 × 10⁵ cells/well/4 ml) for RNA isolation or Western blot experiments. After culture for 24 – 48 h, medium was removed and replaced with an equivalent volume of serum-free media containing 0.1% BSA and the various experimental treatments as indicated. For the analysis of MAPK phosphorylation status, cells were cultured overnight under serum-free conditions prior to stimulation in order to lower the level of basal MAPK phosphorylation. Kinase inhibitors dissolved in stock DMSO solutions were applied 1 h before exposure to MALP-2 or Ni. Untreated control groups received equivalent amounts of DMSO (0.1% final concentration) as vehicle controls. Cells were then returned to the incubator for the indicated times prior to harvest and processing for the selected endpoints.

Analysis of mRNA expression

Total cellular RNA was harvested using RNeasy™ Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instruction and cDNAs generated from 0.5 µg total RNA by reverse transcription as previously described (Gao *et al.*, 2004). Quantitative real-time

reverse transcription-polymerase chain reaction was performed with 5 μ l of cDNA product as previously described (Gao *et al.*, 2004). Briefly, specific primer pairs for IL-6 (forward 5'-GCCAGCTATGAAGCTCTTCTC; reverse 5'-GACTTGTCATGTCCTGCAGCC) and β -actin (forward 5'-GGGACCTGACCGACTACCTC; reverse 5'-GGGCGATGATCTTGATCTTC) were used to amplify the specific cDNAs. PCR reactions were carried out for 20 sec at 95 °C, 30 sec at 55° C, and 40 sec at 72° for 17 cycles for β -actin and 24 cycles for IL-6. PCR products were either detected on 2% agarose gels stained with ethidium bromide or quantified in real-time fashion during the PCR amplification using the double-stranded DNA fluorescent dye PicoGreen™ (Molecular Probes, Eugene, OR) at 430 nm emission and 525 nm excitation in a MJ Reaserch Opticon 2 thermal cycler. Relative gene expression was calculated using the 2- $\Delta\Delta$ Ct methods (Livak and Schmittgen, 2001), using β -actin as the internal control gene to normalize the data for the amount of RNA added to each RT reaction.

Cytokine Analysis

Conditioned medium from treated cells was collected at the indicated time points and centrifuged 400 $\times$ g for 10 min. Supernatants were transferred to new tubes and stored at -20°C until assay. IL-6 content of conditioned media was measured using a specific enzyme-linked immunoassay employing anti-human IL-6 DuoSet™ antibodies (R&D Systems, Minneapolis, MN) as previously described (Gao *et al.*, 2004). Independent analysis of DNA content (Cesarone *et al.*, 1979) after the various experimental treatments revealed no significant changes in cell number and, therefore, validates the presentation of the IL-6 data on a concentration (pg/ml) basis.

Western Blots

For immunological assessment of MAPK, Akt, and COX-2 protein, cell lysates were prepared using a modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate and 1 mM EDTA) containing a cocktail of protease (200 mM PMSF, 1mg/ml leupeptin, aprotinin and pepstatin) and phosphatase inhibitors (200 mM each Na₃VO₄ and NaF), and were clarified by centrifugation at 14,000 $\times$ g. Cell lysates used for HIF-1 α expression were prepared in 10 mM Tris (pH 7.4) with 1% SDS containing the protease and phosphatase inhibitors described above. Protein concentration was determined using the Bradford Assay with bovine serum albumin as standard. Cell lysates (30 μ g protein/lane) were subjected to electrophoresis using NuPAGE™ 4–12% Bis-Tris gels (Invitrogen™, Carlsbad, CA) under denaturing and reducing conditions. Proteins were transferred to Polyscreen® PVDF Transfer membranes (PerkinElmer, Wellesley, MA). Immunoblots were blocked with 5% non-fat dried milk in Tris-buffered saline containing 1% Tween-20 (TBS-T) for 1 h then incubated with anti-phospho-p38 (1:1000), anti-phospho-JNK/SAPK (1:1000), or anti-phospho-ERK_{1/2} (1:1000), anti-phospho-Akt (1:1000) antibodies (Cell Signaling Technology, Danvers, MA), anti-COX-2 (1:000)(Cayman Chemical, Ann Arbor, MI) in TBS-T containing 5% BSA, or anti-HIF-1 α (1:500) in TBS-T containing 5% milk overnight at 4°C. Blots were then rinsed with TBS-T and incubated with anti-rabbit or anti-mouse HRP-linked secondary antibodies (1:2500) for 1 h at room temperature. Immunoblots were developed using LumiGLO® Chemiluminescent Substrate (Millipore, Billerica, MA) and protein was detected by exposure of blot to Lumi-Film Chemiluminescent Detection Film (Roche Applied Science, Indianapolis, IN). Membranes were then stripped with 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris, pH 6.8 for 30 min at 50°C and reprobed for total p38 MAPK, SAPK/JNK or p44/42 MAPK, or Akt (1:1000) (Cell Signaling Technology) or α -tubulin or GAPDH as indicated for loading controls.

Transient transfection of siRNA

Double-stranded siRNA sequences targeting HIF-1 α (HIF1A) (GenBankTM/EBI accession numbers NM_001530, NM_181054) and COX-2 (PTGS2) (GenBankTM/EBI accession number NM_000963) mRNAs were obtained from Dharmacon (Lafayette, CO). A non-targeting siRNA (Dharmacon) was used as a control, and did not significantly affect HIF-1 α and COX-2 mRNA levels compared with non-transfected controls. HLF were cultured in 12-well tissue culture plates (8.9×10^4 /well) and allowed to attach for one day. Cells were transfected for 48 h with 50 nM of HIF-1 α , COX-2, or non-targeting siRNA control (NTC) in OptiMEM using DharmaFECT[®]1 siRNA Transfection Reagent (Dharmacon) according to manufacturer's instructions. After the 48 h transfection period, cells were stimulated with Ni and MALP-2 for 48 h in serum-free MEM containing 0.1% BSA. Levels of IL-6 in the conditioned medium were determined as described above. Real-time RT-PCR and immunoblot analysis confirmed knockdown of Ni and MALP-2-induced HIF-1 α and COX-2 mRNA and protein expression, respectively (data not shown).

Prostaglandin E₂ analysis

Conditioned media samples were collected and stored as described for cytokine analysis. PGE₂ content of conditioned media was measured using a competitive enzyme monoclonal immunoassay (EIA) from Cayman Chemical Co. (Ann Arbor, MI) according to the manufacturer's directions. Multiple dilutions were run in order to insure that values would fall along the linear portion of the standard curve prepared using PGE₂ standard provided with the kit. All reagents and wash buffers were made using UltraPure water (Cayman).

RESULTS

Synergistic interactions between NiSO₄ and MALP-2 to induce IL-6 mRNA and protein production

Our previous study (Gao et al., 2004) in which we observed the synergistic interactions between chemical (ROFA, NiSO₄) and microbial stimulation was restricted to times following 24 and 48 h of concurrent exposure. In order to more carefully delineate the time course and correlate the temporal relationship between changes in IL-6 mRNA gene and protein expression, we measured IL-6 mRNA in cells and IL-6 protein in media at varying times after simultaneous exposure to NiSO₄ (200 μ M) and MALP-2 (600 pg/ml). Figure 1A shows a typical gel of IL-6 and β -actin RT-PCR products obtained from HLF. IL-6 mRNA was barely detectable in unstimulated samples and both Ni and MALP-2 alone produced a measurable response of approximate equivalent magnitude. The combination of Ni and MALP-2 together, however, produced a robust mRNA signal that was much greater than either single stimulus alone at all the selected time points. IL-6 mRNA levels were quantified using real-time RT-PCR and normalized to the expression of β -actin. Figure 1B shows that the Ni/MALP-2 combination produced an early, approximately 12-fold increase in IL-6 mRNA that was substantially greater than the 3 – 4 fold increases seen with MALP-2 and Ni alone at this same time. This early increase was transient with a consistent fall in mRNA at the 4 h time point. As time progressed between 4 and 30 h, however, a second prolonged and persistent accumulation of IL-6 transcripts was observed only in the cells exposed to Ni/MALP-2 combination while IL-6 mRNA in response to either agent alone remained unchanged compared to control.

When IL-6 protein in conditioned media was measured by ELISA (Figure 2), similar levels of IL-6 accumulated over time in response to Ni alone and MALP-2 alone. Each single agent provided significant stimulation when compared to non-stimulated control cells. For example, after 30 h of stimulation IL-6 levels reached 287 ± 71 pg/ml and 352 ± 148 pg/ml for Ni and MALP-2, respectively compared to only 26 ± 6 pg/ml for untreated control cells.

The rate of IL-6 release was much greater when cells were exposed to both Ni and MALP-2 concurrently relative to each agent alone. The inset of Figure 2 compares the observed rate of release in response to Ni + MALP-2 to that predicted by a hypothetically additive model obtained by summation of the responses to each agent alone at each time point. Regression analysis of the resultant lines indicated that IL-6 release in response to combined MALP-2 and Ni was synergistically amplified above that predicted by algebraic summation of the individual responses. While significantly more IL-6 was measured at all time points for the combined stimulation, the most robust enhancement of IL-6 release offered by combined exposure occurred after 8 h of stimulation and correlated with late prolonged phase of mRNA accumulation seen in Figure 1B.

The synergistic stimulation of IL-6 expression by Ni and MALP-2 may require simultaneous presence of both stimulants. Alternatively, the observed delay between exposure and manifestation of maximal synergy suggests that one stimulus may condition cells to respond to subsequent challenge with the other stimulus. To test if single exposure to either agent could modulate the subsequent response to the other we pretreated cells with either NiSO₄ alone, MALP-2 alone, or no addition for 24 h. After 2 washes with PBS to remove the first stimulus, the medium was then replaced with fresh serum-free media (w/0.1% BSA) containing either no addition, NiSO₄ alone, MALP-2 alone or NiSO₄ + MALP-2 and IL-6 content of the media was measured 24 h later. Figure 3 shows that NiSO₄-pretreated cells produced significantly more IL-6 in response to MALP-2 than did Ni-free cells. In these Ni-pretreated cells, secondary stimulation with NiSO₄ plus MALP-2 caused larger production of IL-6 than did MALP-2 alone. In contrast, pretreatment with MALP-2 produced no significant changes in IL-6 production in response to subsequent challenge with NiSO₄, MALP-2 or both, where responses resembled those seen in control non-pretreated cells. These data suggest that NiSO₄ exposure can pre-condition cells to respond more vigorously to subsequent stimulation with MALP-2 for IL-6 production. In contrast, pre-exposure to the TLR-2 agonist did not alter Ni-dependent signaling mechanisms that ultimately lead to IL-6 release.

Time-dependent activation of MAPK and PI3K pathways by Ni and MALP-2

We next sought to more clearly define the molecular mechanisms by which exposure to NiSO₄ potentiates the effects of MALP-2 on IL-6 production. MAPK/SAPK and PI3K are known to play a crucial role in many aspects of immune-mediated and inflammatory responses. Therefore, we chose to focus on these elements of signal transduction. We first determined whether NiSO₄ could activate various MAPK family members. For this we measured the phosphorylation status of ERK_{1/2}, p38 and JNK MAPK isoforms by Western blot after exposure to NiSO₄ (200 μM) for various periods of time. Cells were rendered quiescent by overnight culture in serum-free media (MEM w/0.1% BSA) in order to reduce any serum-induced activation. Figure 4 shows a typical experiment documenting the short-term temporal changes in the levels of protein phosphorylation of ERK_{1/2}, JNK/SAPK, and p38 following exposure to NiSO₄ alone for up to 4 h. Ni induced a rapid and transient phosphorylation of ERK_{1/2} and JNK/SAPK in HLF. ERK_{1/2} and JNK/SAPK activation showed similar kinetics with enhanced phosphorylation observed at 10 and 30 min following Ni exposure (earliest time points measured) and returning to baseline levels by 1 h. No change in the total level (unphosphorylated and phosphorylated forms) of ERK_{1/2} and JNK was observed over this same time course. In contrast, very little or no p38 phosphorylation was detected after short-term Ni stimulation in spite of the fact that total p38 protein was easily observed.

Figure 5 shows a similar analysis for cells treated with MALP-2 alone or the combination of MALP-2 + NiSO₄. MALP-2 alone also rapidly increased phosphorylation of ERK and JNK/SAPK at the 10 and 30 min time points that decreased in intensity after 1 h exposure. When

Ni and MALP-2 were combined we could not detect any substantial differences in the patterns of phosphorylation compared to either stimulus alone. The apparent prolongation of ERK activation at the 60 min time point was not consistently observed in replicate experiments. In a manner similar to that observed with Ni, no activation of p38 phosphorylation could be observed with MALP-2 alone or combined Ni and MALP-2 over the first 4 h of exposure (data not shown).

Given the fact that Ni and MALP-2 interactions seemed to require a significant period of time of exposure to produce amplification of IL-6 release, it was important to also assess the phosphorylation status of MAPKs/SAPKs at later times. Analysis of ERK_{1/2} and JNK/SAPK phosphorylation at 24 and 48 h revealed no significant differences between any of the experimental treatments and control cells (data not shown). Figure 6 shows that p38 activation could indeed be observed but only after a rather prolonged exposure. Exposure to Ni alone produced a small amount of phosphorylated p38 apparent at both the 24 and 48 h exposure time points. Exposure to MALP-2 alone failed to induce p38 activation at either time point, however, the concurrent exposure of Ni and MALP-2 consistently produced greater p38 phosphorylation at the 48 time point compared to Ni alone. Thus, Ni and MALP-2 alone both have the ability to rapidly activate ERK and JNK/SAPK, but not p38, signaling pathways over short-term exposures. This short term activation was transient with restoration to normal levels within 4 h. No consistent interactions between Ni and MALP-2 could be observed when co-exposure was compared to either agent alone over these short times. In contrast, p38 activation was observed only after prolonged exposure to Ni and addition of MALP-2 substantially enhanced this Ni-dependent activation.

The immediate downstream target of PI3K action is Akt. Upon activation of PI3K the resultant second messenger, phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P₃) binds to Akt facilitating its translocation to the membrane. This permits its subsequent phosphorylation on the conserved residues Ser⁴⁷³ and Thr³⁰⁸ via additional protein kinases such as phosphoinositide-dependent kinase (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2). Using phosphospecific antibodies to Akt, we next probed whether Ni and/or MALP-2 modulated the activation of this important regulatory pathway. Figure 7 shows a time course of Akt Ser⁴⁷³ phosphorylation in HLF cultured in the presence and absence Ni and/or MALP-2. At the 1 h time point very little PI3K phosphorylation could be detected regardless of the condition. Significant phosphorylation of Akt, however, was apparent by 6 h and this elevation was maintained throughout the 48 h incubation. The time-dependent increase in Akt phosphorylation was observed even in untreated cells and no significant differences could be detected between any of the various exposure groups. Figure 7 shows a small apparent decrease at 48 h in untreated cells, however, this observation was not reproduced in all experiments and longer times points were not studied. Therefore, it appears that Akt is constitutively phosphorylated on Ser⁴⁷³ in HLF even in the absence of Ni and MALP-2 exposure. The lack of signal seen at the early 1 h time point, mostly likely reflects the media change to serum-free conditions used for the in vitro exposure protocol. Appearance of Akt phosphorylation by 6 h under these conditions suggests that HLF-derived autocrine factors can serve to initiate and maintain this level of Akt activation. We were unable to detect any phosphorylation on Thr³⁰⁸ using antibodies specific for this epitope under basal or Ni- and MALP-2-exposed conditions although robust phosphorylation of both Ser⁴⁷³ (Fig. 7A) and Thr³⁰⁸ (data not shown) were detected in response to PDGF (50 ng/ml, 10 min).

We next wanted to know if Akt phosphorylation in HLF is dependent on PI3K. To this end we examined Akt phosphorylation in the presence and absence of the PI3K inhibitor, LY294002. Figure 7B shows the robust constitutive expression of Ser⁴⁷³-phosphorylated Akt in untreated cells, which again was unaltered by exposure to Ni and/or MALP-2. The

presence of 10 μ M LY294002, however, completely abrogated the phosphorylation of Akt in HLF under all exposure conditions indicating that the constitutive level of Akt phosphorylation is dependent upon the activity of PI3K.

Inhibition of MEK_{1/2}/ERK, p38, and PI3K, but not JNK/SAPK, pathways reduces the ability of NiSO₄ to potentiate MALP-2-induced IL-6 production

Given that Ni and MALP-2 could activate multiple MAPKs in HLF we next tested if pharmacological blockade of specific MAPK-dependent signaling pathways could modulate the interaction between Ni and MALP-2 on IL-6 production. We used four pharmacologic agents with established activity as specific MAPK inhibitors. PD98059 (20 μ M) and U0126 (1 & 5 μ M) specifically target the immediately upstream ERK-activating kinases, MEK₁ and MEK_{1/2}, respectively (Alessi *et al.*, 1995; Favata *et al.*, 1998). SB203580 (20 μ M) (Cuenda *et al.*, 1995) and SP600125 (10 & 20 μ M) (Bennett *et al.*, 2001) were chosen as specific inhibitors of p38 and JNK/SAPK, respectively. Figure 8 (panels A & B) shows the ability of both MEK inhibitors to attenuate IL-6 release in response to 48 h co-exposure to Ni and MALP-2. U0126 (5 μ M) significantly attenuated Ni/MALP-2-induced IL-6 by 56%. In addition, 1 μ M U0126 also reduced IL-6 release by nearly half although this failed to achieve statistical significance. The second MEK inhibitor, PD98059, also inhibited IL-6 release although the degree of inhibition was slightly less (40% inhibition) than that achieved with U0126. This might be expected since U0126 possesses inhibitory activity against both MEK₁ and MEK₂ isoforms, whereas PD98059 is relatively specific for MEK₁.

Inhibitors directed against the two classes of stress-activated MAPKs, SP600125 and SB203580, were next used to probe the role of JNK and p38 MAPKs, respectively. Figure 8 shows that IL-6 release induced by 48 h Ni/MALP-2 co-exposure was resistant to inhibition in the presence of the JNK inhibitor (panel C), but was attenuated by approximately 50% by inclusion of the p38 antagonist (panel D).

It was observed in the course of our western blot experiments for MAPK phosphorylation (Figs. 4 – 6) that Ni and MALP-2 alone could both activate various MAPKs, including ERK. The experiment shown in Figure 3, however, implies that only Ni pre-exposure sensitizes HLF IL-6 expression to a subsequent exposure to the TLR-2 agonist, even when Ni has been removed from the external media. Therefore, we again determined the effects of MAPK inhibitors, but limited their application to a specific phase of the sequential exposure protocol (Ni pretreatment phase or MALP-2 challenge phase). Figure 9 shows that the effects of MAPK inhibition were dependent upon the phase at which they were applied. HLF first treated with Ni for 24 h and then challenged with MALP-2 for 24 h release 713 ± 92 pg/ml of IL-6 during the MALP-2 challenge phase (control bar). This response was attenuated by approximately 50% by the p38 and MEK1 inhibitors (although the effects of PD98059 failed to reach statistical significance), but only when these agents were present during the Ni-pretreatment phase. Similar concentrations present during the MALP-2 challenge did not effect on IL-6 release. Thus, ERK and p38 pathways appear to relate more to the signaling mechanisms modulated by Ni rather than those utilized by MALP-2.

Although we could not detect any changes in the activation of PI3K via monitoring the phosphorylation of its downstream substrate Akt, we did observe that HLF express a significant level of constitutive PI3K activity even under serum-depleted conditions (Fig 7). Therefore, it is possible that this basal activity could exert a permissive role on IL-6 release in response to combined Ni and MALP-2 exposure. Figure 10 demonstrates that inclusion of LY294002 reduced Ni/MALP-2-dependent release of IL-6 to only 29% of that seen in the absence of the PI3K inhibitor. Thus, it appears that the interactive effects of Ni and MALP-2 in coordinating IL-6 release from HLF involve multiple signaling pathways that include, ERK and p38 MAPKs, as well as PI3K.

Impact of kinase-dependent signaling on HIF-1 α and COX-2

In our previous studies dissecting the signaling mechanisms responsible for Ni and MALP-2 synergistic amplification of CXC chemokines, such as IL-8/CXCL8, we observed fundamental roles for hypoxia-inducible factor 1 α (HIF-1 α) and cyclooxygenase 2 (COX-2) in mediating IL-8 release from HLF (Brant and Fabisiak, 2008; Brant and Fabisiak, 2009). It was important, therefore, to determine if Ni and MALP-2 interact via similar mechanisms to direct IL-6 release. To this end, we functionally negated the influence of HIF-1 α and COX-2 by transfection of siRNAs specific for each transcript and determined the effects on Ni/MALP-2 induced IL-6 release. Validation of specificity and the extent of gene “knock-down” by these constructs has been previously described (Brant and Fabisiak, 2009). Figure 11 shows that depletion of either HIF-1 α or COX-2 by siRNA partially inhibited the Ni/MALP-2 induced release of IL-6. A robust induction of IL-6 release by Ni/MALP-2 co-exposure could be observed when cells were transfected with a non-targeting control siRNA (NTC). Negation of either HIF-1 α or COX-2 reduced IL-6 release by a similar amount (40% or 30%, respectively, of response in NTC-transfected cells). Thus, IL-6 released in response to Ni and MALP-2, is at least partially dependent on HIF-1 α and COX-2 similar to our observations with IL-8/CXCL8.

In order to determine if MAPKs or PI3K were involved on the upstream activation of HIF-1 α and COX-2 signaling, we measured whether the various kinase inhibitors could alter the accumulation of HIF-1 α protein and subsequent induction of COX-2 expression that we previously observed in HLF co-exposed to Ni and MALP-2 (Brant and Fabisiak, 2008; Brant and Fabisiak, 2009). Figure 12A shows the effects of U0126 and SB203580 on HIF-1 α accumulation and COX-2 expression in HLF treated with combined Ni and MALP-2. We have previously shown that Ni and Ni/MALP-2 induces a rapid (within 4 h) accumulation of HIF-1 α protein in HLF which is maintained for at least 48 h in the exposed cells (Brant and Fabisiak, 2009). Figure 12 shows the marked accumulation of HIF-1 α observed after 24 h treatment with Ni + MALP-2 which was maintained up to through the 48 h time point. Inclusion of U0126 resulted in a small reduction of HIF-1 α at the 24 h time point, while the amount of HIF-1 α was somewhat reduced in Ni-MALP-2 exposed cells after 48 h of treatment in the presence of both inhibitors. The effect of Ni + MALP-2 on increase COX-2 protein expression can again clearly be seen at the 48 h time point. The inclusion of the SB203580 markedly attenuated COX-2 expression at this time point. U0126 also decreased COX-2 expression although to a lesser extent compared to that seen with the p38 inhibitor. The effect of Ni and MALP-2 on COX-2 is relatively delayed in that only faint expression of COX-2 can be seen at 24 h and this appeared to be unaffected by drug treatment. Increases in PGE₂ could be detected at the 24 h time point, despite the relatively faint expression of immunoreactive COX-2 protein and continued to rise throughout the exposure period. Both U0126 and SB203580 produced striking decrements in the Ni/MALP-2-dependent production of PGE₂ where levels attained after 48 h were reduced by more than 95% compared to that seen in the absence of MAPK inhibitors.

Figure 13 shows the effects of the PI3K inhibitor, LY294002, on HIF-1 α accumulation and COX-2 induction. COX-2 protein expression as assessed by western blot (panel A) was again highly induced following a 48 h co-exposure to Ni and MALP-2, but not when HLF were treated with either stimulus alone. The induction of COX-2 protein was accompanied by a corresponding increase in PGE₂ recovered from medium conditioned by these cells (panel B). The presence of LY294002 during the exposure, however, greatly attenuated the amount of COX-2 protein expressed by Ni/MALP-2 treated cells and concomitantly reduced the level of PGE₂ release by over 90%. Exposure of Ni alone was sufficient to produce accumulation of HIF-1 α regardless of whether MALP-2 is present and this response was insensitive to inhibition of PI3K (Fig. 13A).

DISCUSSION

This study extends our exploration into the potential mechanisms underlying the synergistic interactions between Ni and microbial toxins, such as MALP-2. Our data are consistent with the hypothesis that Ni exposure amplifies subsequent TLR-2-dependent cell activation and cytokine release in HLF. Similar to our observations with several CXC chemokines (Brant and Fabisiak, 2008; Brant and Fabisiak, 2009), we now show that IL-6 also shares HIF-1 α and COX-2 as part of the signal transduction pathway mediating amplified release during Ni and MALP-2 exposure. In addition, we demonstrate that specific protein kinase signaling pathways, most notably p38, PI3K, and ERK_{1/2} are also involved in this interaction and begin to organize their role within the framework of HIF-1 α and COX-2-dependent signaling.

While Ni levels in air average 1 – 2 ng/m³ over much of the US geographic area, 1 million and 200 million live in the vicinity of Ni point sources producing 200 ng/m³ and 50 ng/m³, respectively (Leikauf, 2002). This fact is underscored by the fact that ambient levels approaching 50 ng/m³ are routinely recorded in the winter months in New York City (Peltier *et al.*, 2009). We previously estimated that breathing an ambient level of 100 ng/m³ over a 24-hr period could theoretically produce 1 μ M concentration of Ni within the extracellular space of the lung (Gao *et al.*, 2004). Given that the threshold for Ni-induced synergy with MALP-2 is 5 – 10-fold lower than the 200 μ M used here (Gao *et al.*, 2004) heterogeneous distribution, altered breathing patterns, and/or time-dependent accumulation might all contribute to local concentrations sufficient to recapitulate the effects seen here. Moreover, other atmospheric hypoxia-mimetic metals might enhance IL-6 release. To our knowledge, no one has ever measured the level of MALP-2 or other toll-like receptor agonists during infection, however, we should point out that in our hands MALP-2 is approximately 1000-fold more potent than the bacterial lipopolysaccharide (LPS) frequently used to model activation of innate immune mechanisms (Brant and Fabisiak, 2009), and that similar synergistic interactions can be observed using a sub-cytopathic inoculum of live *Mycoplasma fermentans* (Gao *et al.*, 2004).

IL-6 is a pleiotropic cytokine with inflammatory (Ohshima *et al.*, 1998), immune-modulatory (Diehl *et al.*, 2002), as well as protective (Ward *et al.*, 2000), and growth promoting (Roth *et al.*, 1995) effects. IL-6 is readily induced in various lung cells exposed to PM (Quay *et al.*, 1998; Gao *et al.*, 2004; Zhao *et al.*, 2009) and IL-6 deficiency attenuates lung injury and inflammation following cigarette smoke and ozone exposure (Yu *et al.*, 2002). Exposure of humans to elevated levels of PM increases circulating levels of IL-6 (Ruckerl *et al.*, 2007) while rodent studies have linked IL-6 levels to PM-induced thrombosis (Mutlu *et al.*, 2007).

The time course of IL-6 gene expression following co-exposure to Ni and MALP-2 revealed a biphasic induction of mRNA accumulation over the 30 h time period studied. It is clear that synergistic interactions between Ni and MALP-2 participate in both phases of mRNA accumulation. This implies that regulation of IL-6 gene expression by Ni and MALP-2 is complex and may impact multiple signaling events. In spite of the bimodal pattern of IL-6 mRNA expression, however, the robust increase in IL-6 protein most closely correlated with the second phase of induction, perhaps because of the extended time period over which the high mRNA levels were maintained.

It appears that both stimuli do not need to be present simultaneously for interactions to occur, as evidenced by our sequential addition experiments. Pre-exposure to Ni facilitated subsequent MALP-2 stimulation of IL-6 rather than a reciprocal augmentation of Ni signaling by MALP-2. We cannot, however, fully exclude the possibility that trace amounts

of Ni remain within the cell even after vigorous washing and contribute to the response. We have previously observed a similar effect of facilitation of MALP-2-dependent IL-6 release following pre-exposure of HLF to TNF- β , an extracellular protein ligand that is likely more confidently removed with washing (Fabisiak *et al.*, 2006). The addition of Ni (or TNF) along with the MALP-2 challenge, however, does further enhance the response indicating that maximal effects can be better realized with concurrent exposure to both agonists.

The importance of various protein kinase-dependent signaling pathways in the regulation of inflammatory and innate immune responses has been documented many times over, however, often with conflicting results. For example, inhibition of PI3K activity in human periodontal ligament cells unanimously inhibited IL-6, IL-8, and M-CSF release in response to periodontal pathogens (Dommish *et al.*, 2008; Guan *et al.*, 2009), but markedly amplified IL-6 released by osteoblasts following PDGF-BB (Hanai *et al.*, 2006). In addition, while p38 has usually been considered as a positive mediator of inflammatory cytokine release including IL-6, at least one report contests this dogma by showing that SB203580 attenuates the caveolin-dependent inhibition of macrophage-derived IL-6 (Wang *et al.*, 2006). Thus, it is challenging to delineate any generalized role for these signaling mechanisms in IL-6 expression as their roles are undoubtedly highly stimulus- and cell type-specific, require strict temporal coordination, and depend on the presence or absence of ancillary signaling pathways.

Exposure to Ni alone induced rapid and pronounced phosphorylation of both ERK_{1/2} and JNK/SAPK. This action, however, was also evident in response to MALP-2 alone and co-exposure of Ni + MALP-2 failed to further augment the degree MAPK activation. Thus, Ni and MALP-2 do not interact to facilitate ERK activation per se and ERK activation alone is not responsible for augmenting IL-6 release since neither Ni nor MALP-2 treatment alone approximated the levels of IL-6 produced during co-exposure. None the less, the inhibitory influence of both MEK_{1/2} inhibitors on IL-6 release following Ni and MALP-2 co-exposure points to the importance of this pathway in certain settings. Our data indicate minimal involvement of JNK/SAPK in Ni/MALP-2 interactions on HLF-derived IL-6.

PI3K is fundamentally important for Ni and MALP-2 interactions since inhibition of this pathway produces an even greater decrement in IL-6 than that seen with inhibition of MEK_{1/2}. Interestingly, none of the applied stimuli (Ni, MALP-2, Ni + MALP-2) produced changes in PI3K signaling as reported by AKT phosphorylation above that seen in untreated control cells. Thus, again Ni and/or MALP-2 alone do not impact the degree of PI3K activation per se yet PI3K activity is permissive for optimal Ni/MALP-2 interactions. At the earliest time point (1 h) after media change and application of stimuli, levels of Akt phosphorylation were very low in all treatment groups including control, but rose significantly after 6 h and were maintained throughout the incubation. This “basal” level of phosphorylation requires PI3K since Akt phosphorylation is completely abrogated by the PI3K inhibitor, LY294002. These findings suggest that constitutive activation of PI3K-dependent signaling is required for full realization of Ni and MALP-2 interactions. The mechanisms accounting for this basal level of PI3K activation remains elusive, but data are consistent with a role for HLF-derived autocrine factors such as IGF-1 with capacity to stimulate PI3K (Moats-Staats *et al.*, 1993; Doepfner *et al.*, 2007).

At the moment we can only speculate as to the roles of ERK_{1/2} and PI3K on Ni/MALP-2-induced IL-6 release, but they most likely require the presence of additional pathways to manifest their influence. Both ERK (Tuyt *et al.*, 1999; Jiang *et al.*, 2004) and PI3K (Ozes *et al.*, 1999; Sizemore *et al.*, 1999) have been shown to amplify the transactivating capacity of NF- κ B through a variety of mechanisms. IL-6 expression is also driven by additional transcriptional regulators including AP-1 and Sp1 (Gerlo *et al.*, 2008). Using BEAS-2B

cells, Andrew et al. observed that Ni-induced AP-1 was required for full activation of the HIF-1 α -dependent expression of PAI-1 (Andrew *et al.*, 2001). In contrast, we recently showed that Ni increased Sp1, as well as HIF-1 α , levels in an ERK-dependent manner but that the majority of Ni-induced VEGF was Sp1-dependent (Nemec and Barchowsky, 2009). ERK and p38 may also act to induce the stabilization of IL-6 mRNA (Andoh *et al.*, 2002; Zhao *et al.*, 2008).

Here we show that Ni-induced accumulation of HIF-1 α is important for the synergistic interactions of Ni and MALP-2 on IL-6. Although similar to what we have previously observed with IL-8 (Brant and Fabisiak, 2009), the degree of attenuation of IL-6 following application of siRNA is only about half of what we observed for IL-8. For the moment we do not know if IL-6 gene represents a direct target for transcriptional activation by HIF-1 α or if IL-6 can be modulated secondarily by another HIF-1 α gene product such as COX-2 (Kaidi *et al.*, 2006). RNA interference of HIF-1 α attenuated IL-6 induced in endothelial cells in response to hypoxia-mimetic stimuli, however, the putative HRE region identified by chromatin immunoprecipitation was located quite far upstream of the transcriptional start site (approx -10,000 bp) and its functionality was not directly tested (Viemann *et al.*, 2007).

Ni is a well-known hypoxia-mimetic stimulus that results in HIF-1 α stabilization, although the precise mechanism is controversial. Ni may directly target and inhibit prolyl hydroxylase (Davidson *et al.*, 2006) or deplete intracellular ascorbate (Salnikow *et al.*, 2004), a necessary co-factor of prolyl hydroxylase. HIF-1 α is also a direct target for ERK and PI3K action. ERK activation only modestly enhanced HIF-1 α levels in BEAS-2B cells in response to particulate (Andrew *et al.*, 2001) and soluble forms (Nemec and Barchowsky, 2009) of Ni, but was shown to be a major player in determining its transactivating potential following these stimuli. Apparently, ERK-dependent phosphorylation can enhance its nuclear localization (Mylonis *et al.*, 2008), as well as mediate its association with other co-effectors of the transcriptional apparatus like p300 (Sang *et al.*, 2003). Similarly, experimental loss-of-function or gain-of-function manipulations of PI3K can, respectively, decrease or increase HIF-1 α -dependent gene expression in response to growth factors and other stimuli (Zhong *et al.*, 2000; Zundel *et al.*, 2000; Jiang *et al.*, 2001). In our studies, inhibition of PI3K or MEK_{1/2} had minimal effect upon HIF-1 α protein levels following Ni or Ni + MALP-2 stimulation. Therefore, if ERK_{1/2} or PI3K exert direct effects on HIF-1 α they presumably do so via other post-translational modifications of its transactivating potential.

Soluble Ni can activate all three MAPK isoforms in skin- (Boisleve *et al.*, 2004a) and blood-derived (Aiba *et al.*, 2003) dendritic cells where it has been studied in light of its role as a contact sensitizer. For the most part, p38 appears critical for this metal's ability to induce the majority of the endpoints of dendritic cell maturation (Arrighi *et al.*, 2001; Boisleve *et al.*, 2004b). We did not observe substantial p38 activation in response to Ni alone, perhaps because of the lower concentrations employed in our studies. The mixture of Ni and MALP-2, however, produced a pronounced phosphorylation of p38 but only after prolonged exposure. This pattern in many ways mirrors the interactive effects of Ni and MALP-2 on COX-2 and cytokine release. COX-2 expression was markedly attenuated and PGE₂ release nearly completely blocked in the presence of the SB203508 implying that p38 acts upstream of COX-2. p38 has been implicated in COX-2 induction following chemical stressors such as acrolein and zinc (Wu *et al.*, 2005; Park *et al.*, 2007). One possible pathway is via phosphorylation and activation of Sp1 as was shown by Xu et al. during EGF induction of COX-2 in glioma cells (Xu *et al.*, 2009).

The existence of a PGE₂-dependent positive feedback loop also means that PGE₂ can further modify the same initial downstream events that initially triggered the responses. PGE₂ itself has been shown to stabilize HIF-1 α and drive HIF-dependent expression of VEGF in a

human prostate cancer cell line (Liu *et al.*, 2002). COX-2 can itself be auto-amplified in a PGE₂-dependent manner, a process which is in part dependent on p38 activation (Suda *et al.*, 1998; Faour *et al.*, 2001). Elements of PGE₂-dependent signaling have also been linked to activation of ERK, as well as PI3K (Fujino *et al.*, 2003; Han and Wu, 2005; Yu *et al.*, 2008), although the specific receptors and mechanisms for this may differ between cell types. Thus, it is also possible that Ni and MALP-2 had significant impact on cellular events that pertain to the COX-2- and prostanoid-dependent amplification of IL-6. Current studies in our lab are addressing the ability of Ni and MALP-2 to impact PGE₂-dependent signaling events and the role of specific PGE₂ receptor subtypes in mediating the interactions between chemical and microbial stress. Of note is the fact that Ni alone can switch a normally suppressive role of PGE-2 on IL-8 synthesis to a stimulatory role (Brant and Fabisiak, 2009).

It should be noted that appreciable Ni/MALP-2 induction of COX-2 expression could still be observed in the presence of various kinase inhibitors, especially U0126, despite nearly complete blockade of PGE₂ production. Furthermore, significant PGE₂ was readily detected in 24 h conditioned media samples from Ni/MALP-2 treated cells, although COX-2 protein was only barely detectable. These data suggest that other steps in eicosanoid biosynthesis, such as phospholipase A₂ and prostaglandin E synthase-1 might also be subject to regulation during Ni and MALP-2 co-exposure.

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Figure 1. NiSO₄ and MALP-2 co-exposure induces synergistic increases in IL-6 gene expression
After seeding into 6-well plates and culture for 48 h, HLF were exposed to NiSO₄ (200 μM) and/or MALP-2 (600 pg/ml) under serum-free conditions (MEM w/0.1% BSA). At the indicated time points total cellular RNA was isolated. IL-6 mRNA expression was then assessed by regular and quantitative RT-PCR as described in METHODS. Panel A shows the representative PCR products corresponding to IL-6 and β-actin mRNAs observed from a typical experiment visualized by ethidium bromide staining. Panel B shows the results of quantitative real-time PCR performed in the presence of PicoGreen™ for IL-6 mRNA normalized to β-actin. Data represent mean ± SEM from 5 experiments each performed with a cell line derived from a different donor. Data from each specific time point were analyzed by one-way repeated measures ANOVA and Newman-Keuls multiple comparisons test. *Denotes statistically significant difference ($p < 0.05$) of MALP-2 plus Ni combined group when compared to both the Ni alone and MALP-2 alone groups for a given time point. #Denotes statistically significant difference between Ni alone and MALP-2 alone.

Figure 2. Time course of IL-6 release by HLF in response to NiSO₄, MALP-2, and NiSO₄ plus MALP-2

Cells were seeded and stimulated as described in Figure 1. At the indicated time points the conditioned media were recovered and IL-6 measured by ELISA as described in METHODS. Data represent mean $\pm$ SEM from 5 experiments each performed with a cell line derived from a different donor. Data from each specific time point were analyzed by one-way repeated measures ANOVA and Newman-Keuls multiple comparisons test.

*Denotes statistically significant difference ($p < 0.05$) of MALP-2 plus Ni combined group when compared to both the Ni alone and MALP-2 alone groups for a given time point. Inset compares the observed rate of IL-6 release following Ni + MALP-2 to that theoretically predicted from a simple model of additivity based on effects of each agonist alone. The slopes calculated by linear regression are statistically different from one another and indicative of a synergistic interaction.

Figure 3. NiSO₄ pretreatment facilitates subsequent MALP-2-induced IL-6 release in HLF
 HLF were pre-exposed to MALP-2 (600 pg/ml) or NiSO₄ (200 μM) or no MEM w/0.1% BSA. Control cells received medium alone (None). Medium was then removed 24 h later and each pre-exposure condition then received fresh medium alone (white bars) or media containing MALP-2 (gray bars) or NiSO₄ (hatched bars) alone or together (black bars) for an additional 24 h at which time medium was collected and analyzed for IL-6. Data represent mean ± SEM obtained from 6 replicate experiments each using a different single donor-derived cell line. Arrows denote statistically significant differences between indicated groups along with significance level assessed by repeated measures one-way ANOVA and Bonferroni's Multiple Comparison test.

Figure 4. Transient activation of ERK_{1/2} and JNK/SAPK by NiSO₄

HLF were plated into p60 dishes in complete media and grown for 2 days until confluent. Prior to experiment cells were rendered quiescent by overnight culture in serum-free medium (MEM plus 0.1% BSA) and then treated with 200 μ M NiSO₄ for the indicated times. Total cell lysates were prepared, electrophoresed, transferred to PVDF membrane and immunoblotted with phospho-specific antibodies directed towards activated ERK_{1/2}, JNK, or p38 MAPKs (Cell Signaling Technologies, Danvers, MA). Following chemiluminescent detection of the signal the blots were stripped and then reprobed using primary antibodies that recognize both phosphorylated and non-phosphorylated forms to measure total amount of each MAPK protein in the sample. GAPDH served as a loading control. Data are representative of at least 3 independent experiments performed using a composite HLF cell line derived from multiple donors.

Figure 5. Transient activation of ERK_{1/2} and JNK/SAPK during treatment with MALP-2 and Ni/MALP-2

HLF were treated with MALP-2 (600 µg/ml) or combination of NiSO₄ (200 µM) and MALP-2 after overnight culture in serum-free media. Total cell lysates were prepared at the indicated times and western blots performed for phosphorylated and total ERK and JNK/SAPK. Data are representative of at least 3 independent experiments performed using a composite HLF cell line derived from multiple donors.

Figure 6. Activation of p38 MAPK after prolonged co-exposure to Ni and MALP-2
 HLF were treated as described in METHODS and then exposed to NiSO_4 (200 μM) alone, MALP-2 (600 pg/ml) alone, or Ni + MALP-2 combined. At 24 and 48 h after treatment total cell lysates were prepared and subjected to western blotting using primary antibodies directed towards phosphorylated and total p38. Membranes were then reprobbed using anti-GAPDH antibody as a loading control. Data are representative of at least 3 independent experiments performed using a composite HLF cell line derived from multiple donors.

Figure 7. PI3K-dependent phosphorylation of Akt in control and Ni/MALP-2 treated HLF
 A. HLF were treated with MALP-2 (600 pg/ml), NiSO₄ (200 μ M), or both together as described in MATERIALS and METHODS. At the indicated times, whole cell lysates were prepared and Ser⁴⁷³ phospho-Akt was assessed by western blot. Positive and negative controls represent HLF treated in the presence (+) or absence (-) of PDGF for 10 min, respectively. Expression of α -tubulin was used as a loading control. Amounts of total (phosphorylated and non-phosphorylated) Akt were unchanged when probed using a pan-Akt antibody (data not shown). B. Treatment of HLF with the PI3K inhibitor LY294002 (10 μ M) blocks Akt phosphorylation. LY294002 was applied to cells 1 h prior to exposure to Ni and/or MALP-2. Western blots were performed for phospho-Akt, total-Akt, and α -tubulin

were performed after 48 h exposure to Ni and/or MALP-2. Data shown are representative of 3 independent experiments performed using a composite HLF cell line.

Figure 8. Effect of various MAPK inhibitors on IL-6 release during concurrent exposure to MALP-2 and NiSO₄

A pooled composite line of HLF was treated with MALP-2 (600 pg/ml) and NiSO₄ (200 μ M) together in the presence or absence of indicated MAPK inhibitors. MAPK inhibitors were given 1 h prior to Ni and MALP-2 exposure and were present throughout the incubation. Conditioned medium was collected 48 h after stimulation and analyzed for IL-6 by ELISA. U0126 (panel A) and PD98059 (panel B) were chosen as inhibitors of MEK, the activating kinase immediately upstream of ERK_{1/2}. SP600125 (panel C) and SB203580 (panel D) were chosen to target JNK/SAPK and p38 MAPKs, respectively. Data represent mean $\pm$ SEM from 3 – 5 experiments performed using a single composite multiple donor-

derived cell line. Control cells represent Ni/MLALP-2-treated cells in the absence of inhibitors. *Denotes statistically significant difference ($p < 0.05$) relative to control in the absence of any inhibitor by either paired t-test (B & D) or repeated measures one-way ANOVA and Dunnett's Multiple Comparisons to Control (A & C).

Figure 9. Blockade of MEK/ERK_{1/2} and p38 MAPKs during Ni-pretreatment, but not MALP-2 challenge, reduces MALP-2-induced IL-6 release

HLF were pre-exposed to NiSO₄ (200 μM) in MEM w/0.1% BSA for 24 h. Medium was then removed, cells washed, and fresh medium containing MALP-2 (600 pg/ml) was applied for another 24 h. Half of the Ni-pretreated cells received one of three MAPK inhibitors (PD98059, SB203580, or SP600125) only during the Ni-pretreatment phase followed by MALP-2 in the absence of drug. Parallel wells with cells pretreated with Ni received MAPK inhibitors only during the MALP-2 challenge and IL-6 collection phase. Inhibitors were all given 1 h before Ni or MALP-2 exposure and at a final concentration of 20 μM. Control cells represent Ni-pretreated cells challenged with MALP-2 in the absence of inhibitors

during either phase. Data represent mean $\pm$ SEM of observations collected from 6 experiments each using a unique HLF cell line derived from a different donor. *Denotes statistically significant difference between indicated groups by repeated measured one-way ANOVA and Dunnett's Multiple Comparisons to Control ($p < 0.05$).

Figure 10. Blockade of PI3K with LY294002 attenuates IL-6 release in response to Ni and MALP-2 co-exposure

HLF were cultured and treated as described in METHODS. Cells were treated with MALP-2 (600 pg/ml) plus NiSO₄ (200 μ M) in the presence and absence of LY294002 (10 μ M). LY294002 was applied one hour prior the addition of Ni and/or MALP-2 and remained throughout the incubation. After 48 h conditioned media were removed and IL-6 content measured. Control cells represent Ni and MALP-2 treated cells in the absence of LY294002. Data represent mean $\pm$ SEM for 3 experiments using a single pooled cell line composed of HLF derived from 5 different donors. *Denotes statistically significant difference from control ($p < 0.05$) by paired Student's t-test.

Figure 11. Negation of HIF-1 α and COX-2 expression by siRNA partially blocks Ni/MALP-2-induced IL-6

HLF were cultured and transiently transfected with siRNA constructs specific to human COX-2, HIF-1 α , or a non-targeting control sequence (NTC) as described in METHODS. Cells were then switched to serum-free conditions and treated with Ni + MALP-2 in serum-free medium containing 0.1% BSA for 48 h. Control cells received serum-free medium with 0.1% BSA alone. Conditioned media were collected 48 h after Ni/MALP-2 exposure and IL-6 measured by ELISA. Data represent mean $\pm$ SEM obtained from 2 separate experiments done in triplicate performed using a composite cell line pooled from 5 different donors. Degree of target knockdown was established using real-time RT-PCR and western blots (data not shown) and was equivalent to that observed by us in previous studies (Brant and Fabisiak, 2009). *Denotes statistically different from non-targeting control (NTC) by one-way ANOVA and Dunnett's Multiple Comparison to Control ($p < 0.05$)

Figure 12. Effects of U0126 and SB203580 on COX-2 and HIF-1 α expression and PGE₂ release by HLF in response to Ni and MALP-2

HLF were cultured in 6-well plates and treated as described in METHODS. Cells were exposed to Ni + MALP-2 (200 μ M and 600 pg/ml, respectively) or left untreated in the presence or absence of U0126 (10 μ M) or SB203580 (20 μ M). MAPK inhibitors were applied 1 h prior to Ni and MALP-2 treatment and remained throughout the incubation. No inhibitor groups received an equivalent amount of DMSO (0.1%) as vehicle control. Panel A shows typical western blots for COX-2 and HIF-1 α expression after 24 and 48 h of Ni/MALP-2 exposure. Alpha-tubulin was included as a loading control. Data shown are representative of a typical experiment repeated at 3 times using a pooled composite cell line. Panel B shows the content of PGE₂ contained in the conditioned media. Data represent mean $\pm$ SEM obtained from 3 experiments. Groups were compared by one-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test. *Denotes significant difference compared to untreated control cells at the same time point. #Denotes significant difference relative to Ni/MALP-2 treated cells in the absence of any MAPK inhibitor at that same time point.

Figure 13. Effects of LY294002 on COX-2 and HIF-1 α expression and PGE₂ release by HLF in response to Ni and MALP-2

HLF were cultured and treated with NiSO₄ (Ni, 200 μ M), MALP-2 (M, 600 pg/ml) or both together (NiM) for 48 h as described in METHODS. Some cells were treated in the presence of the PI3K inhibitor, LY294002 (10 μ M), added 1 h prior to addition of Ni and/or MALP-2 and present throughout the incubation. Cells treated in the absence of LY294002 received a similar amount of DMSO alone (0.1%) to control for any effects of the solvent vehicle alone. Panel A shows western blot analysis of COX-2 and HIF-1 α protein expression following Ni and MLAP-2 stimulation in the presence and absence of inhibitor. Alpha-tubulin was included as a loading control. Data shown are representative of three independent experiments using a pooled composite cell line. Panel B shows the PGE₂ content of conditioned media from Ni and MALP-2 treated cells in the presence and absence of inhibitor analyzed using specific enzyme-linked immunoassay. Data represent mean $\pm$ SEM obtained from 3 independent experiments. ***Significantly different from no inhibitor within respective treatment group (Bonferroni post hoc test, $p < 0.001$).