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MYELOPEROXIDASE MODULATES LUNG EPITHELIAL RESPONSES TO PRO-INFLAMMATORY AGENTS

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ABSTRACT

During extensive inflammation, neutrophils undergo secondary necrosis causing myeloperoxidase (MPO) release which may damage resident lung cells. Recent observations suggest that MPO has pro-inflammatory properties, independent of its enzymatic activity. The aims of this study were to characterize MPO internalization by lung epithelial cells and to investigate the effect of MPO on oxidative stress, DNA damage and cytokine production by lung epithelial cells.

Human alveolar and bronchial epithelial cells were stimulated with MPO with or without priming the cells with pro-inflammatory stimuli. MPO protein was detected in cell cytoplasm. Expression of heme-oxygenase (HO)-1 and DNA strand breakage were determined. The production of interleukin (IL)-8 and 6 were measured.

Analyses of MPO stimulated cells demonstrated MPO presence in the cells. HO-1 expression was increased after MPO stimulation and increased further when cells were primed before MPO stimulation. MPO exposure also induced DNA strand breakage. Interestingly, MPO inhibited IL-8 production in bronchial, but not alveolar epithelium.

In conclusion, alveolar and bronchial epithelial cells can internalize MPO. Stimulation with MPO increases HO-1 expression and DNA strand breakage, suggesting cell damaging capacity of MPO. In addition, MPO inhibited IL-8 production by bronchial epithelial cells, indicating a negative feedback loop for neutrophil recruitment.

INTRODUCTION

Neutrophilia is a feature common to a number of inflammatory lung diseases including chronic obstructive pulmonary disease (COPD) [1], asbestosis [2], idiopathic pulmonary fibrosis [3] and adult respiratory distress syndrome [4] although the agents and events leading to the development of these diseases are very different. Independent of the cause of the chemotactic gradient, neutrophils are activated during their recruitment to the lung and release the contents of their granula into the pulmonary compartment. Excess amounts of neutrophils can lead to secondary necrosis of neutrophils as a result of inefficient clearance of apoptotic neutrophils by macrophages. During secondary necrosis, neutrophils release their lysosomal constituents which possibly affect resident lung cells [5].

Currently, over 50 neutrophil derived-toxins have been identified, including proteolytic enzymes and bacteriacidal proteins. The heme protein, myeloperoxidase (MPO), is the most abundant protein in neutrophils and represents 5% of their total protein content [6]. MPO is synthesized as a precursor during myeloid differentiation in bone marrow, and its processing is completed before neutrophils enter the circulation [7-9]. A hallmark of neutrophil activation is their respiratory burst in which MPO plays a central role [10]. MPO catalyses the conversion of hydrogen peroxide (H_2O_2) and chloride ions into hypochlorous acid (HOCl) and therefore it is an important enzyme in the host defence against bacteria, viruses and fungi [10]. MPO may also damage tissue by its production of HOCl and other reactive oxidants. HOCl production by MPO leads to the formation of chlorotyrosine (ClTyr) residues, a marker of neutrophilic inflammation [11]. Nitric oxide (NO) and nitrite (NO_2^-) also serve as biological substrates of MPO leading to the formation of nitrogen dioxide (NO_2). NO_2 promotes protein nitration, lipid peroxidation and the oxidation of tyrosine yielding nitrotyrosine (NO_2Tyr), a post-translational modification and a contributor of inflammatory disease [12]. In addition to its role as a catalytically active protein generating reactive

oxidants, MPO recently has been shown to elicit pro-inflammatory properties independent of its enzymatic activity. MPO was demonstrated to associate with the outer membrane of neutrophils by binding to CD11b/CD18 integrins, which are known to be linked to neutrophil activation [13]. In this manner, MPO may contribute to neutrophil recruitment to sites of inflammation. In addition, MPO stimulates the production of tumor necrosis factor α (TNF-α) and interferon α/β (IFN-α/β) by peritoneal macrophages and thereby enhances their killing of target cells [14]. Whether MPO released from neutrophils affects resident lung cells and their responses in the absence or presence of an initial inflammatory trigger is unknown.

Reactive oxidant species can be produced by MPO and since HO-1 is an indirect marker of oxidative stress, we hypothesized that MPO exposure would lead to HO-1 up-regulation and an increase in DNA cell damage. Neutrophil recruitment is induced by the production of chemoattractants at an inflammatory site therefore the production of IL-8 and IL-6 by lung epithelial cells was investigated.

Here we used bronchial and alveolar epithelial cells under basal (homeostatic) and primed conditions. These primed conditions were created by stimulating cells with lipopolysaccharide (LPS) to mimic lung infection, with asbestos to mimic asbestosis and phorbol 12-myristate 13-acetate (PMA) was used as positive control. The aims of our study were to 1) characterize MPO internalization by lung epithelial cells and 2) to investigate the effect of MPO on oxidative stress, DNA damage and IL-8 and 6 production by lung epithelial cells under basal and inflammatory conditions. Our results demonstrate that alveolar and bronchial epithelial cells internalize MPO. Stimulation with MPO increases HO-1 expression and DNA strand breakage, suggesting that MPO is capable of damaging lung epithelial cells. In addition, MPO inhibits IL-8 production by bronchial epithelial cells, indicating that MPO may be involved in a negative feedback loop for the recruitment of neutrophils.

MATERIALS AND METHODS

Cells and stimulation: Human bronchial epithelial cells (Beas-2B) and a human transformed alveolar epithelial cell line (A549) were obtained from the ATCC and cultured in RMPI 1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Biochrome, Berlin, Germany), L-Glutamine (2 mM) and Penicillin/Streptomycin (both Invitrogen, Grand Island, NY). Tissue culture surfaces for the Beas-2B cells were coated with LHC basal medium containing 10% bovine serum albumin (both Biosource International, Camarillo, CA), 1mg/ml fibronectin and 2.9mg/ml bovine collagen, Type 1 (both BD Biosciences, Bedford, MA). Cells were plated at a density of 20,000 cells/cm^2 for analyses of IL-6 and IL-8 production, and at a density of 13,000 cells/cm^2 for immunofluorescence staining on coverslips and Western blot analysis. 18-24 hours prior to stimulation growth medium was replaced by medium containing 0.5% FBS. LPS (*Escherichia coli,* 055:B5; Sigma, St. Louis, MO), PMA (Sigma, St. Louis, MO) and MPO (Calbiochem, San Diego, CA) stocks were prepared and stored at -20°C. National Institute of Environmental Health Sciences (NIEHS) processed crocidolite and chrysotile asbestos was always used fresh. Asbestos was weighed, UV-sterilized, suspended in 1mg/ml in tissue culture medium and triturated 8x through a 22-gauge needle to obtain a homogeneous suspension. When LPS, PMA or asbestos stimulation was followed by MPO stimulation, media from controls and pre-stimulated cells was replaced with fresh MPO containing RPMI 1640 with 0.5% FBS. Supernatants were harvested and stored at -20°C until used for ELISA.

MPO detection: *Immunofluorescence staining in vitro* Cells were washed 2x with ice-cold PBS followed by fixation with 4% paraformaldehyde for 5 minutes. Cells were permeabalized with 100% ice-cold methanol for 3 minutes followed by two washes with PBS and blocking with 1% (w/v) BSA in PBS for 30 minutes. Incubation with rabbit anti-human MPO (0398,

DAKO, Glostrup, Denmark) in 0.1% (w/v) BSA in PBS for 60 minutes was followed by incubation with goat anti-rabbit Texas Red (4010-07, Southern Biotechnology Associates, Birmingham, AL) for 30 min in the dark. Nuclei were stained with diamidino-2-phenylindole. Negative controls by omitting primary antibody revealed no staining.

Immunohistochemistry on lung sections: Peripheral lung specimens of patients undergoing lung surgery were processed immediately following resection by fixation in 10% phosphate-buffered formalin prior to embedding in paraffin. To verify that epithelial cells are affected by released MPO lung sections (3µm) from COPD or asbestosis patients were cut and deparafinized 3x 5 min in microclear, followed by 2x 5 min 100% ethanol. Endogenous peroxidase was blocked by incubation in 0.6% H_2O_2 in methanol for 15 minutes followed by hydrating through ethanols (80%, 70%, 50%) to water. The sections were equilibrated in Tris-buffered saline (TBS) (pH 7.6) for 5 minutes followed by 30 minute blocking with 20% normal swine serum in TBS-Tween20 (0.01%) with 1% (w/v) BSA. A rabbit anti-human MPO (0398, DAKO) primary antibody diluted in TBS-Tween20 (0.01%) with 1% (w/v) BSA was incubated for 45 minutes followed by 30 minutes incubation with a swine anti-rabbit biotinylated secondary antibody (E0413, DAKO) and avidin-biotinylated horseradish peroxidase (K0377, DAKO). Enzymatic reactivity was visualized with HistoGreen (Linaris, Wertheim, Germany). Negative controls by omitting primary antibody revealed no staining.

Preparation of membrane and cytoplasmic fractions: Cells were placed on ice and washed 2x with ice-cold PBS. Cells were suspended in lysis buffer (25mM Tris, pH8/ 2mM $MgCl_2$/ 5mM KCl/ 1mM Na orthovanadate/ 1mM phenylmethyl sulfonyl fluoride/ 10mM NaF/ 10µg/ml Aprotinin/ 10µg/ml Leupeptin), homogenized and vortexed for 10 seconds. Cell nuclei were spun down for 10 min at 1000 g at 4°C. Supernatant was transferred to a new tube, and the membrane fraction was spun down for 30 min at 14,000 rpm at 4°C.

Cytoplasmic fractions were transferred to new tubes and the membrane fractions were resuspended in lysis buffer. A portion of both fractions was saved for protein determination, prior to the addition of 4x Laemmli sample buffer (0.25M Tris-HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4M DTT and 0.04% (w/v) Bromophenol Blue). Next, samples were boiled for 5 minutes and stored at -20°C.

Preparation of nuclear extracts: Cells were placed on ice and washed twice with ice-cold PBS. Cells were suspended in cytoplasmic buffer (10mM Tris-HCl; pH 7.6/ 10mM KCl/ 1.5mM $MgCl_2$/ 1% TritonX-100/ 1mM dithiothreitol / 0.2mM Na orthovanadate/ 0.4mM phenylmethyl sulfonyl fluoride/ 10µg/ml Leupeptin/ 0.2mM NaF), vortexed vigorously and placed on ice for 15 min. Then cell nuclei were spun down for 5 min at 10,000g at 4°C. Supernatant was transferred to a new tube and cell nuclei were washed once in cytoplasmic buffer by suspending, vortexing and centrifugation. The supernatant was discarded, and cell nuclei were suspended in nuclear buffer (20mM Tris-HCl; pH7.6/ 0.4mM KCl/ 1.5mM $MgCl_2$/ 10% glycerol/ 1mM dithiothreitol/ 0.2mM Na orthovanadate/ 0.4mM phenylmethyl sulfonyl fluoride/ 10µg/ml Leupeptin/ 0.2mM NaFl). Cells were placed in a vertical rotator at 4°C for 30 minutes followed by centrifuging for 20 min at 14,000g at 4°C. Supernatant containing the nuclear proteins was transferred to new tube. A portion of the supernatant was saved for protein determination, prior to the addition of 4x Laemmli sample buffer (0.25M Tris-HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4M DTT and 0.04% (w/v) Bromophenol Blue). Next, samples were boiled for 5 minutes and stored at -20°C.

Western Blot analysis: Protein concentrations were determined using the Bradford protein Assay according to manufacturer's protocol (Bio-Rad, Hercules, CA). Protein (10µg for MPO and AP-1, 5µg for p65) was loaded per lane and separated on a polyacrylamide gel (Mini

Protean 3 System, Bio-Rad), followed by transfer to a 0.45μm nitrocellulose membrane (Bio-Rad) by electroblotting. The membrane was blocked for 1 hour at room temperature in 5% (w/v) non-fat, dried milk diluted in TBS-Tween20 (0.05%). Nitrocellulose blots were washed in TBS-Tween20 (0.05%), followed by overnight (o/n) incubation (4°C) with primary antibody (rabbit anti-MPO (0398, DAKO); rabbit anti-p65 (C-20 sc-372, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho c-Jun (Ser63) II (9261, Cell Signaling, MA)). After 3 washes of 20 minutes each, the blots were probed with HRP-conjugated anti-rabbit antibody (PI-1000, Vector Laboratories, Burlingame, CA) and visualized by chemiluminesence using Supersignal®WestPico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) according to manufacturers' instructions and exposed to film (Biomax light film, KODAK).

Quantitative PCR detection of HO-1 and IL-8 expression: Whole cell RNA was isolated using the RNeasy Mini Kit (Qiagen Inc., CA) according to manufacturer's protocol. Total RNA was reverse transcribed, using the Abgene Kit (Abgene, Epsom, UK) with random hexamer primers, and the resulting cDNA was amplified by real-time PCR using the MJ Research Opticon 2 (Biorad, Hercules, CA) with the following primers: HO-1 (F) 5′-CCAGCAACAAAGTGCAAGATTC-3′, (R) 5′-CTGCAGGAACTGAGGATGCTG-3′; IL-8 (F) 5' GGACAAGAGCCAGGAAGAAA 3', (R) 5' AAATTTGGGGTGGAAAGGTT 3', β-actin (F) 5′-GGGACCTGACCGACTACCTC, (R) 5′GGGCGATGATCTTGATCTTC. Each PCR reaction contained 1x SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA) and 0.3μM each of forward and reverse primer. Following an initial 10 min incubation at 95°C, thermal cycling was performed using 45 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Gene expression was quantified using standard curves for the

respective cDNA products. All changes in HO-1 and IL-8 cDNA levels were normalized to changes in β-actin cDNA.

Single cell gel electrophoresis (Comet assay): DNA strand breakage in epithelial cell preparations was assayed immediately after cell-isolation by the Comet assay. Fully frosted slides were coated with agarose and stored overnight at 4°C. Epithelial lung cells were isolated by trypsinized, spun down and resuspended in 20µl cold PBS. Cells were mixed with 70µl 0.5% low melting point agarose, and added to the slides, on top of the first agarose layer using a cover glass. Slides were stored at 4°C for 3 minutes to allow solidification, cover glasses were removed and slides were immersed in lysis buffer (2.5M NaCl/ 100mM EDTA/ 10mM Tris-base/ 250mM NaOH; pH 10) and stored overnight at 4°C. The following day, slides were rinsed with distilled water and placed in an electrophoresis tank filled with ice-cold electrophoresis buffer (300mM NaOH/ 1mM EDTA, pH 13) for 20 min. Electrophoresis was conducted at 300mA and 25V for 20 min. Slides were neutralized 3x 10 min using neutralization buffer (0.4M Tris, pH 7.5). All steps after cell lysis were performed in the dark or under dimmed red light to prevent additional DNA damage.

ELISA: IL-6 and IL-8 protein levels in culture supernatants were assayed in triplicate using ELISA kits specific for IL-6 and IL-8 (Sanquin, Amsterdam, the Netherlands) according to the manufacturer's instructions. The lower detection limit of IL-6 was 3.8 pg/ml and 9.0 pg/ml for IL-8.

Statistical analyses: Data are expressed as Mean ± SEM, unless stated otherwise. Statistical comparisons among experimental groups were performed analysis of variance (ANOVA). Differences were considered to be statistically significant when $P \leq 0.05$.

RESULTS

MPO uptake by human bronchial and alveolar epithelial cells: Bronchial (Beas-2B) as well as alveolar (A549) epithelial cells were stimulated with MPO to determine the time frame of MPO internalization by these cells. Immunofluorescense staining demonstrated MPO protein on A549 and Beas-2B cells (Fig 1A) after 24 hours of stimulation. Western blot analysis (Fig 1B) of cytoplasmic protein fractions showed that MPO was detectable in A549 cells after 30 minutes of stimulation and was still internalized after 24 hours of stimulation. In contrast to A549 cells, MPO content in Beas-2B cells peaked after 4 hours of stimulation and was decreased again after 24 hours. Priming of the cells with either LPS or PMA did not have an effect on intracellular levels of MPO (data not shown). To confirm our *in vitro* data on MPO uptake by lung epithelial cells, MPO was localized in human lung sections of patients with pulmonary neutrophilia suffering from COPD, acute broncho pneumonia and asbestosis. Table 1 shows subject characteristics. Figure 1C and D illustrate representative MPO stainings and demonstrates that all cells in close proximity to extravasated and degranulated neutrophils are possibly target cells. Endothelial cells near neutrophils that are not fully activated and have not migrated into the inflammatory site do not contain MPO.

MPO exposure after primary stimulus results in increased oxidative stress: To investigate the influence of MPO on oxidative stress as a result of pro-inflammatory stimuli, cells were stimulated with MPO alone or primed with PMA, LPS, or crocidolite or chrysotile asbestos, followed by stimulation with MPO. MPO exposure of A549 (Fig 2A) and Beas-2B (Fig 2B) cells resulted in an increased HO-1 expression, an indirect marker of oxidative stress. LPS was the only pro-inflammatory stimulus, besides from MPO, which could induce HO-1 expression significantly in A549 cells. MPO stimulation of cells primed with PMA or

asbestos resulted in a significant increase of HO-1 expression as compared to priming control A549 cells (Fig 2A). In Beas-2B cells only asbestos priming resulted in increased HO-1 expression. Asbestos priming followed by MPO stimulation had a synergistic effect on HO-1 expression in Beas-2B cells (Fig 2B).

MPO stimulation increases DNA damage: MPO produces damaging reactive oxidants, like HOCl and NO_2. The COMET assay was used to detect DNA strand break formation in lung epithelial cells after MPO exposure. MPO exposure resulted in a temporary increase in DNA strand breakage in both A549 (Fig 3A) and Beas-2B (Fig 3B) cells, starting 10 minutes after MPO exposure. DNA damage increased significance after 20 min and 30 minutes after MPO exposure the DNA strand breakage started to decline.

IL-8 and IL-6 production are decreased after MPO stimulation of Beas-2B cells: To investigate the influence of MPO on IL-6 and IL-8 production under basal and pro-inflammatory conditions, cells were primed with PMA, LPS, crocidolite or chrysotile asbestos or medium alone, and followed by stimulation with MPO. Figure 4A demonstrates that all pro-inflammatory stimuli caused an increase in IL-8 production by Beas-2B cells. Stimulation with MPO alone or after priming with PMA decreased IL-8 production significantly. These significant results were confirmed by analysis of IL-8 expression levels by Q-PCR (Fig 4B). IL-6 production by Beas-2B cells demonstrated a similar pattern as IL-8, although decreases were not significant (Fig 4C). MPO exposure of A549 cells, under basal and inflammatory conditions, did not influence IL-8 production, and IL-6 production was undetectable (data not shown).

Activation of cell signalling pathways following cell stimulation: The promoter regions of HO-I, IL-8 and IL-6 have binding sites for the transcription factors, AP-1 and NF-κB. To investigate if MPO affects HO-I expression and IL-8 and IL-6 production via the transcription factor AP-1 and NF-κB in lung epithelial cells, A549 and Beas-2B cells were stimulated with LPS, PMA, asbestos or MPO, and AP-1 and NF-κB transcriptional activity was determined by western blot analyses. A 60 min stimulation of A549 cells with MPO did not affect transcriptional activity of either AP-1 or NF-κB (Fig 5A) or Beas-2B cells (data not shown). Increased activity of NF-κB, measured by Rel A translocation, in A549 (Fig 5B) and Beas-2B (data not shown) cells was found after 60 min stimulation with LPS, PMA or crocidolite asbestos.

DISCUSSION

The heme protein, MPO, is the most abundant protein in neutrophils [6] and therefore an important enzyme in innate immunity [10]. More recently, MPO has been shown to elicit pro-inflammatory properties independent of its catalytic properties. We are the first to report here the effects of MPO on human lung epithelial cells and their responses in the absence or presence of an initial inflammatory trigger.

Using two techniques we demonstrate MPO uptake by bronchial and alveolar epithelium after MPO stimulation. Beas-2B cells showed similar staining patterns as small airway epithelial cells, reported by Yang [15], demonstrating MPO in the cytoplasm, but not in the nucleus. In contrast, A549 cells do not show a pronounced lack of nuclear staining. This suggests that MPO distribution after uptake varies between cell types. Neutrophils go through several stages of activation during the extravasation process. The majority of MPO content of neutrophils is released during the oxidative burst of neutrophils when neutrophils are fully activated and have entered a site of inflammation [16]. With immunostaining of human lung tissue from COPD, acute broncho pneumonia and asbestosis patients with active neutrophilia we confirm our *in vitro* data and demonstrate that, not only epithelial cells but all cells in close proximity to activated and degranulated neutrophils are possible target cells for MPO. This is in line with literature describing MPO uptake by other cell types like fibroblasts [17] and endothelial cells [18].

The enzymatic activity of MPO results in the formation of HOCl, which is a potent oxidant with cell damaging capacities. We demonstrate here an increase in lung epithelial cell DNA damage after incubation with purified MPO. This DNA damage is most likely due to HOCl production by MPO. HOCl related normal bronchial epithelial cell damage has been reported before [19] and confirms our data using the Comet assay. In contrast to our findings, a decrease in DNA damage was observed when MPO was added to cell cultures provided with

an H_2O_2 generating system [20]. However, neutrophils and macrophages used in this study [20] produce reactive oxidant species themselves. It can therefore be speculated that these cell types are better protected against oxidative damage than lung epithelium, since lung epithelial cells do probably not produce reactive oxidants at the same magnitude as neutrophils and macrophages [21].

HO-1 is an important cytoprotective enzyme and widely accepted indirect marker of oxidative stress. Our data show an increase in HO-1 expression by bronchial and alveolar epithelial cells after MPO stimulation. We first reported that crocidolite asbestos exposure leads to a significant increase of HO-1 expression in human mesothelial cells [22] , and this has recently been confirmed in an animal model [23]. Here we show that both crocidolite and chrysotile, an iron-free asbestos, stimulate HO-1 expression in Beas-2B cells. More striking was the finding of a synergistic effect of MPO stimulation after asbestos priming on HO-1 expression in both cell lines, which was not demonstrated with LPS or PMA. We speculate that O_2^- or H_2O_2 released by frustrated phagocytosis of long asbestos fibers [24, 25] synergize with nitrosating or oxygen free radicals produced by MPO.

We demonstrate here an increase in IL-8 and IL-6 production, both involved in the development of inflammation, after PMA or asbestos stimulation confirming published literature [26-28]. Here we show for the first time that MPO stimulation of PMA primed Beas-2B cells results in a significant decrease of IL-8 production and a trend towards decreased IL-6 production by Beas-2B cells. Novel as well are our data demonstrating that under basal conditions MPO can inhibit the production of IL-8 by Beas-2B cells. This may indicate a negative feedback loop for neutrophil recruitment. Cytokine production by A549 and Beas-2B cells is a serum dependent event as described previously [29], and because we performed our experiments under low serum conditions, this may explain why we were unable to detect basal production of IL-6 by A549 cells.

Since IL-8 and IL-6 are both known to be NF-κB and AP-1 regulated genes [30, 31], and the HO-1 promoter region contains NF-κB and AP-1 binding sites, we hypothesized that MPO stimulation of A549 and Beas-2B would affect NF-κB or AP-1 transcriptional activity. To our surprise, neither NF-κB nor AP-1 was affected by MPO stimulation, although increases in NF-κB activity were observed with asbestos, LPS and PMA. We have reported previously that both NF-κB and AP-1 activity increases after exposure to crocidolite asbestos [32, Janssen, 1995 #267, 33] and NF-κB activity also has been demonstrated in lung epithelium after instillation of LPS [34]. More research needs to be done to investigate the transcriptional mechanisms underlying HO-1 expression and cytokine production after exposure of lung epithelial cells to MPO. Other candidate transcription factors involved in HO-I expression are NrF2, Bach-1 and HIF-1α [25].

This study puts forward novel mechanisms of MPO action, which may be important in the development of lung neutrophilia. A limitation of the study is however the use of epithelial cell lines, which may have lost some of their *in vivo* properties. Therefore, future studies using primary epithelial cell cultures as well as studies using *in vivo* models of acute lung inflammation will be necessary to investigate the role of MPO in the development of neutrophilic lung inflammation. For example studies using MPO-/- vs. WT mice have demonstrated a role for MPO in a chrysotile asbestos induced model of lung inflammation. The recruitment of neutrophils was significantly delayed in MPO-/- as compared to WT mice [35]. In summary, the present study demonstrates that lung epithelial cells in close proximity to fully activated and degranulated neutrophils are possible target cells for MPO. In these cell types, MPO induced the expression of HO-I and increased DNA strand breakage, but has inhibitory effect on the production of pro-inflammatory chemokines IL-6 and IL-8 during homeostasis and secondary to PMA exposure.

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Table 1: Subject characteristics of patients with acute broncho pneumonia, chronic obstructive pulmonary disease or asbestosis

	AGE, YEARS	GENDER, M/F	SMOKING STATUS	TISSUE COLLECTION
COPD 1	70	M	ex	LVRS
COPD 2	73	M	current	Tumor Resection
COPD 3	66	M	ex	Tumor Resection
ABP 1	75	M	ex	Tumor Resection
ABP 2	72	M	ex	Tumor Resection
Asbestosis 1	51	M	unknown	Tumor Resection
Asbestosis 2	70	M	unknown	Tumor Resection
Asbestosis 3	77	M	unknown	Tumor Resection

ABP: Acute Broncho Pneumonia; M: male; Ex-smoker: quitting smoking for at least 6 months before the start of the study; LVRS: lung volume reduction surgery.

FIGURE LEGENDS

Figure 1: Localization of myeloperoxidase (MPO) in A549 and Beas-2B cells after MPO stimulation and immunohistochemical detection of MPO in human lung sections of patients with chronic obstructive pulmonary disease, acute broncho pneumonia or asbestosis. (A) Positive immunofluorescence staining of MPO on A549 and Beas-2B cells after 24 hours of MPO (40nM) stimulation (top). A549 cells show a diffuse staining pattern, whereas Beas-2B cells show a cytoplasmic staining but no nuclear staining. Untreated A549 and Beas-2B cells do not show MPO staining (bottom). Staining from one representative picture is shown. (B) Western blot analyses of MPO (1nM) stimulated A549 and Beas-2B cells showed that MPO was present in cell cytoplasm 30 minutes after MPO stimulation, but did not accumulate after 24 hours. Representative immunostaining for MPO on human lung sections of (C) chronic obstructive pulmonary disease and (D) asbestosis. Alveolar epithelial cells (➢) in close proximity to degranulated neutrophils stained positive for MPO. Endothelial cells (➢) nearby intravascular un-degranulated neutrophils stained negative for MPO. (A) Internal scale bar = 25μM. (C-D) Internal scale bar = 50μM.

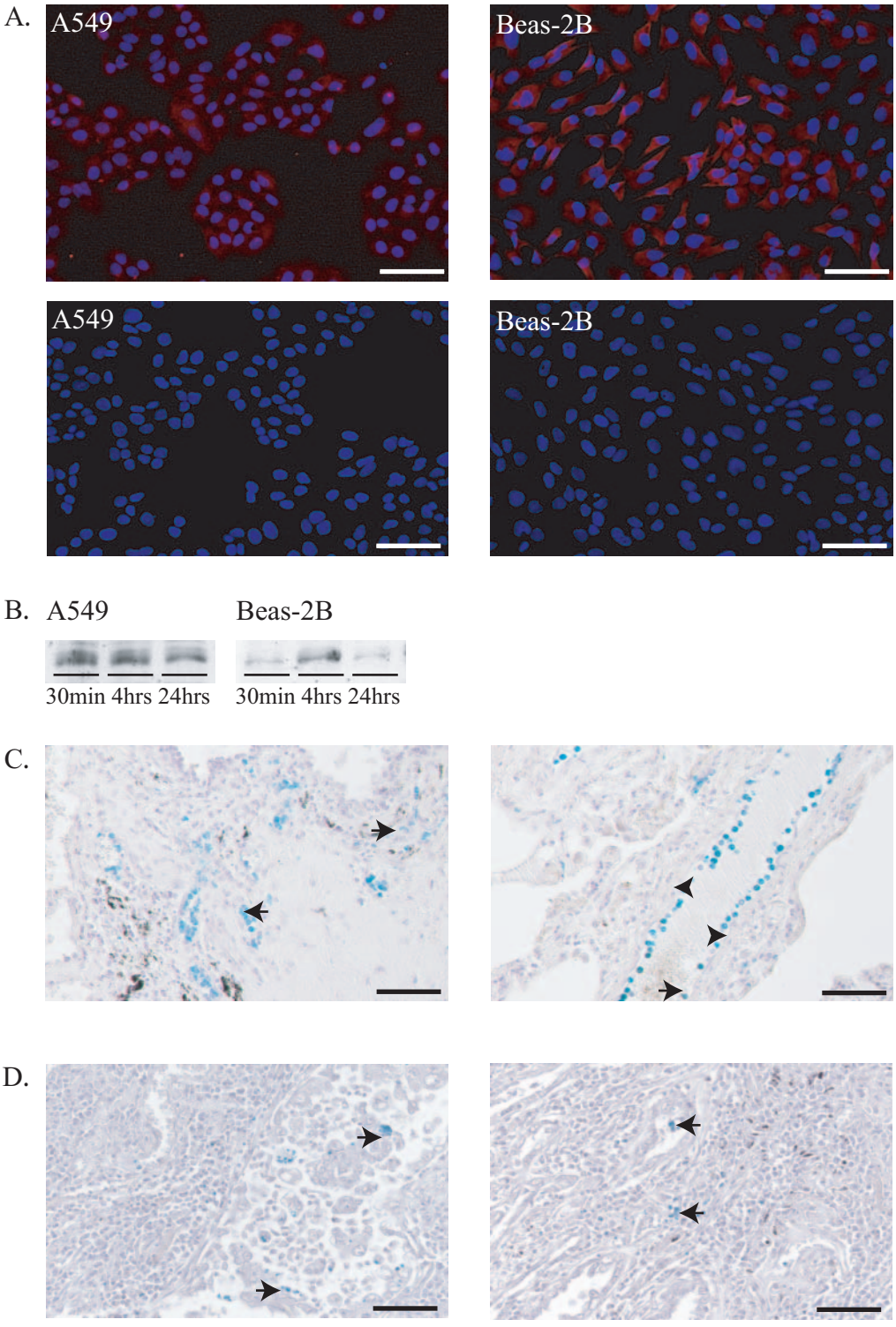


Figure 2: Quantitative PCR analysis of heme-oxygenase 1 (HO-1) messenger RNA after myeloperoxidase (MPO) stimulation in A549 (A) and Beas-2B (B) cells under basal and pro-inflammatory conditions. Cells were stimulated with MPO (10nM) for 24 hours, or primed for 12 hours with lipopolysaccharide (LPS) (3µg/ml), Phorbol 12-myristate 13-acetate (PMA)

(A549:10ng/ml, Beas-2B:20ng/ml) or asbestos (6.25µg/cm^2) followed by 12 hour MPO stimulation. (A) Stimulation with MPO increased HO-1 expression in A549 cells. Stimulation with MPO after pre-stimulation with PMA or asbestos, increased HO-1 expression even further. (B) MPO stimulation of Beas-2B cells resulted in up-regulation of HO-1 expression. Priming the cells for with PMA or asbestos followed by of MPO stimulation resulted in a significant further increase of HO-1 expression. Representative data of 2 individual experiments are shown. Values are normalized against β-actin expression (Mean ± SEM). * = $p \leq 0.05$ *versus* unstimulated cells. † = $p \leq 0.05$ *versus* primed control.

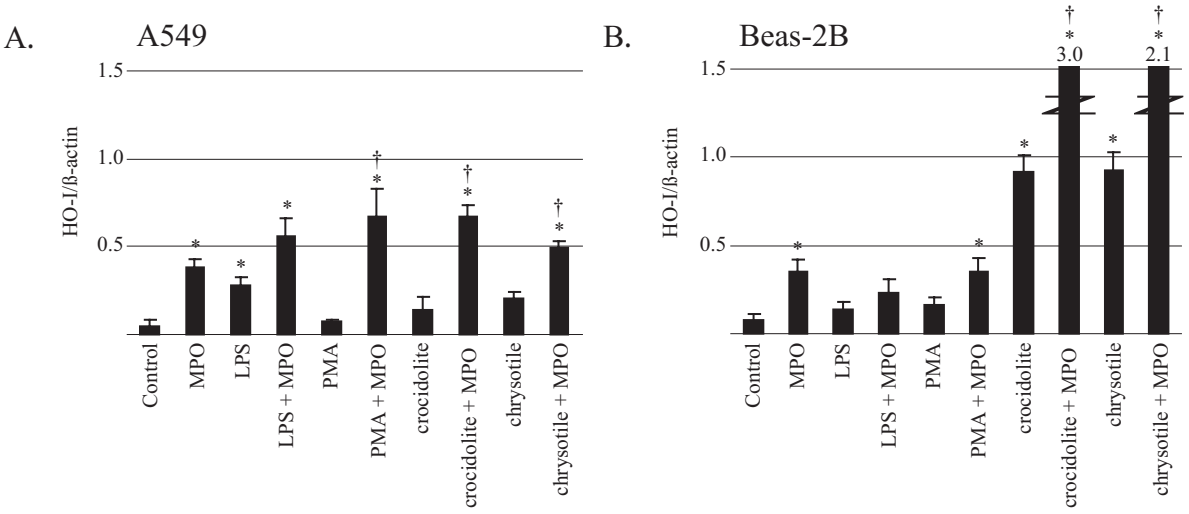


Figure 3: COMET assay analysis of DNA strand breakage after myeloperoxidase (MPO) stimulation in A549 (A) and Beas-2B (B) cells. MPO (40nM) exposure increases DNA strand breakage in both cell lines. A significant increase was reached after 20 min of MPO stimulation, and the level of DNA damage declined thereafter. Representative data of 3 individual experiments are shown. Values are graphed as median tail length (Mean ± SEM). * = $p \leq 0.05$ *versus* unstimulated cells.

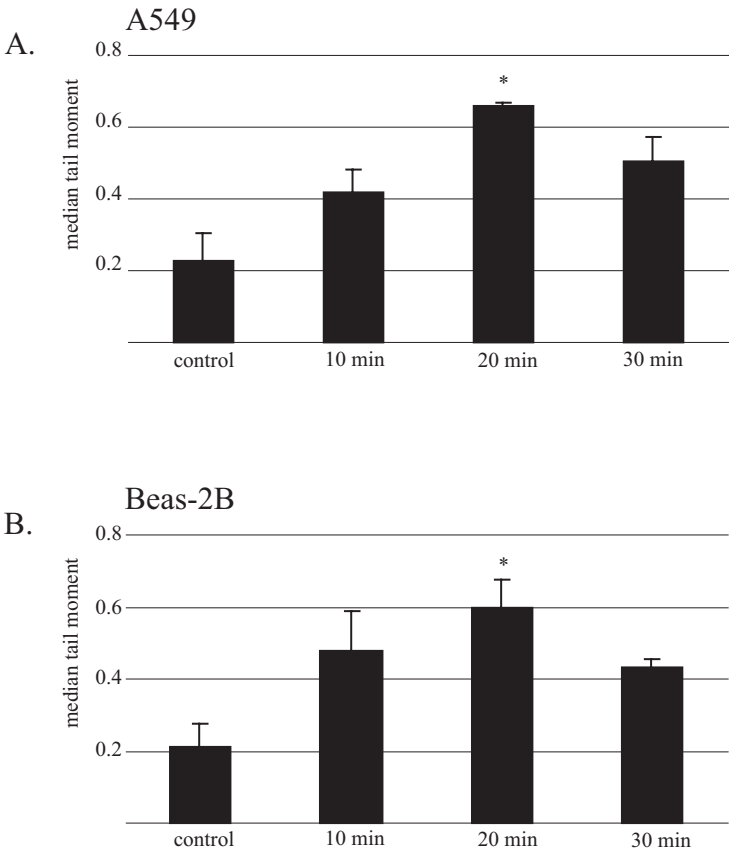


Figure 4: Detection of interleukin (IL)-8 production (ELISA) and expression (Q-PCR) and IL-6 production (ELISA) by Beas-2B cells under basal and pro-inflammatory conditions. Cells were stimulated with myeloperoxidase (MPO) for 24 hours, or primed for 24 hours with lipopolysaccharide (LPS) (3μg/ml), Phorbol 12-myristate 13-acetate (PMA) (A549:10ng/ml, Beas-2B:20ng/ml) or asbestos (6.25μg/cm^2) followed by 24 hour MPO stimulation. (A) IL-8 production by Beas-2B cells is significantly decreased after MPO (40nM) stimulation alone or after priming with PMA followed by MPO stimulation. (B) ELISA results were confirmed by Q-PCR analysis of IL-8 expression after MPO stimulation alone or after priming with PMA followed by MPO stimulation. IL-8 expression levels are normalized against β-actin expression. (C) IL-6 production by Beas-2B cells showed a similar pattern as IL-8, but the decreases were not significant. Croc = crocidolite, Chry = chrysotile. Representative data of 3 individual experiments are shown. Values are graphed as percentage of unstimulated control

(=100%) (Mean ± SEM). * = $p \leq 0.05$ *versus* unstimulated cells. † = $p \leq 0.05$ *versus* primed control.

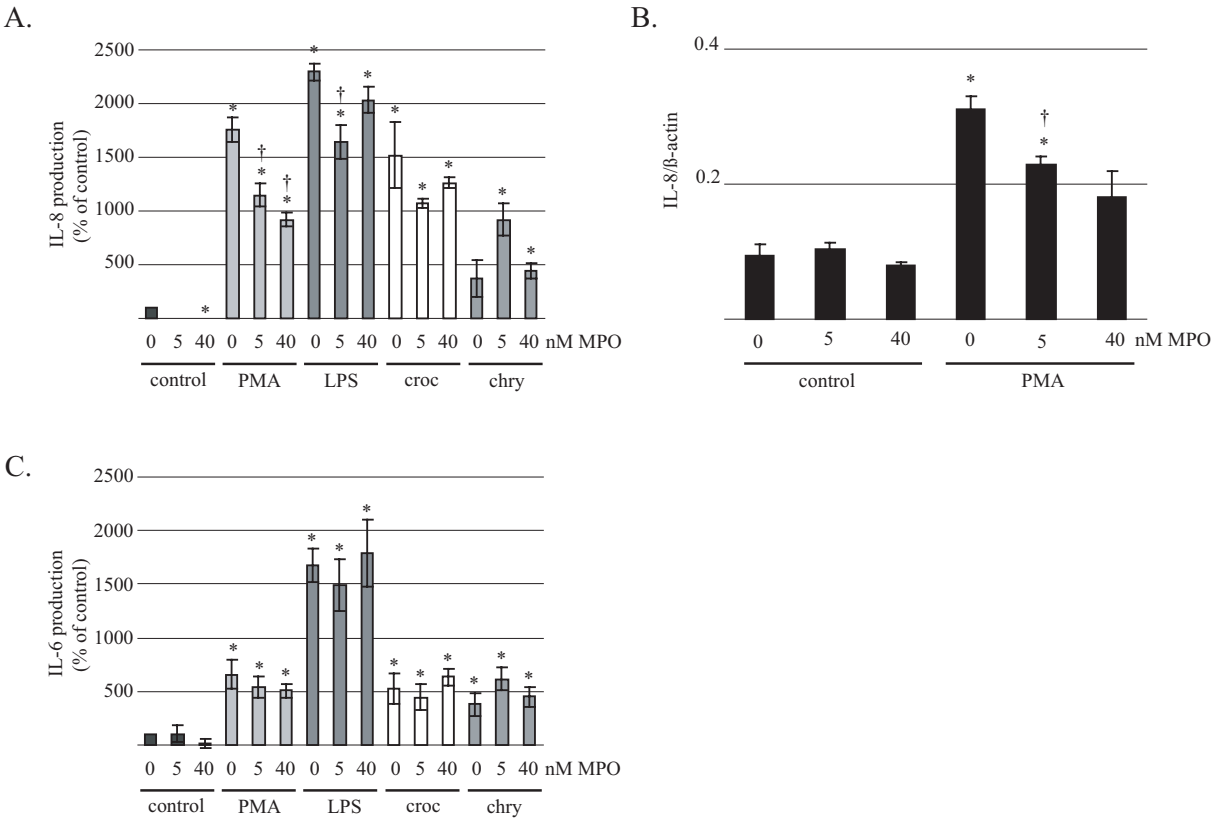
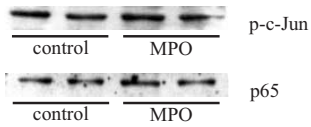
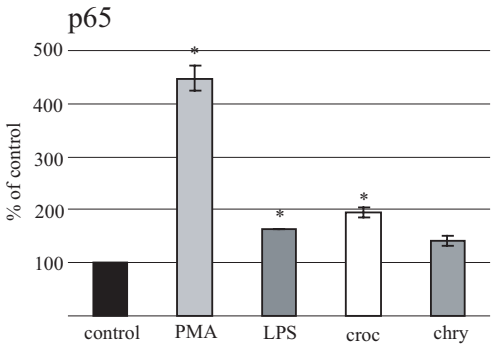


Figure 5: Western blot detection of Rel A and p-c-Jun in A549 cells. (A) Myeloperoxidase (MPO) (10nM) stimulation for 60 min did not increase Rel A translocation or c-Jun phosphorylation in A549 cells. (B) Rel A translocation is increased in A549 cells after 60 min stimulation with Phorbol 12-myristate 13-acetate (PMA) (10ng/ml), lipopolysaccharide (LPS) (3µg/ml) or asbestos (6.25µg/cm2). Representative data of 2 individual experiments are shown. Croc = crocidolite, Chry = chrysotile. Signal intensity was determined with PhosphorImager analysis, and expressed as relative (%) to control. Values are graphed as Mean ± SEM. * = $p \leq 0.05$ *versus* unstimulated cells.

A.


control
MPO
p-c-Jun
control
MPO
p65

B.


p65
% of control
500
400
300
200
100
control
PMA
LPS
croc
chry