Lung inflammation is associated with reduced pulmonary nucleotide excision repair in vivo

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Chronic pulmonary inflammation is associated with increased lung cancer risk, but the underlying process remains unknown. Recently, we showed that activated neutrophils inhibit nucleotide excision repair (NER) in pulmonary epithelial cells in vitro via the release of myeloperoxidase (MPO). To evaluate the effect of neutrophils on NER in vivo, mice were intratracheally instilled with lipopolysaccharide (LPS) (20 μg), causing acute lung inflammation and associated neutrophil influx into the airways. Three days post-exposure, phenotypical NER capacity was assessed in lung tissue homogenate. LPS exposure inhibited pulmonary NER by ~50%. This finding was corroborated by down-regulation of the NER-associated genes Xpa and Xpf. To further elicit the role of neutrophils and MPO in this process, we utilized MPO-deficient mice as well as mice in which circulating neutrophils were depleted by antibody treatment. LPS-induced inhibition of pulmonary NER was not affected by either Mpo−/− or by depletion of circulating neutrophils. This contrasts with our previous in vitro observations, suggesting that inhibition of pulmonary NER following acute dosing with LPS is not fully mediated by neutrophils and/or MPO. In conclusion, these data show that LPS-induced pulmonary inflammation is associated with a reduction of NER function in the mouse lung.

Introduction
Chronic inhalation of combustion-derived particles (e.g., cigarette smoke) is a major determinant in the development of lung cancer, one of the most lethal types of cancer worldwide. A generally accepted explanation for this relationship is the ability of such particles to transport chemical carcinogens into the lung. However, another important characteristic is that the particles elicit an influx of polymorphonuclear neutrophils (PMN) into the lung, which is most evident in smokers who developed chronic obstructive pulmonary disease (COPD) (1). COPD encompasses a local chronic inflammation in the airways (2,3), dominated by invasion of neutrophils, macrophages and T cells (4). Interestingly, smokers diagnosed with COPD have an increased risk of developing lung cancer (3- to 10-fold) even after having quit smoking (5,6). The prominent presence of neutrophils associated with COPD (4,7) suggests a significant role of the ongoing neutrophilic inflammation in this carcinogenic response (8).

Neutrophils have been implicated in (pulmonary) carcinogenicity by their genotoxic capacity. This is thought to occur through the release of mutagenic reactive oxygen species (ROS) leading to oxidative DNA damage, as well as by promoting the metabolic activation of environmental chemical carcinogens via the enzyme myeloperoxidase (MPO), resulting in promutagenic DNA adducts (9). Moreover, we have shown recently that neutrophils, via the MPO-catalysed formation of hypochlorous acid (HOCl), are potent inhibitors of the nucleotide excision repair (NER) pathway in cultured pulmonary epithelial cells, causing a delayed removal of promutagenic bulky DNA adducts (10).

To date, evidence of a role of neutrophils in pulmonary carcinogenicity has been largely derived from in vitro studies using co-cultures of neutrophils and pulmonary epithelial target cells to study neutrophil-induced genotoxicity (9). Evidence from in vivo studies is, however, primarily circumstantial and additional animal studies are necessary to substantiate causality. The aim of the present study was to assess the in vivo effect of neutrophilic inflammation on NER in the mouse lung. Therefore, mice were intratracheally instilled with lipopolysaccharide (LPS), followed by the assessment of neutrophil infiltration, MPO activity and NER capacity in pulmonary tissues. In order to characterize the role of MPO, mice deficient in this enzyme were also subjected to LPS and similar analyses were performed. Furthermore, circulating neutrophils were depleted by intraperitoneal treatment with a neutrophil-specific antibody to provide direct evidence for the relation between neutrophils and NER inhibition in vivo. Our results suggest that LPS-induced acute lung inflammation is associated with a suppression of pulmonary NER, which is not mediated by neutrophil influx and the subsequent release of the enzyme MPO.

Materials and methods

Animals
Male Wt C57Bl6 mice (~12 weeks old) were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Mpo−/− mice were generated by Aratani et al. (11) and bred into the C57Bl6 background. Mice were housed individually in standard laboratory cages and allowed food and water ad libitum throughout the experiments. The studies were carried out in accordance with an approved protocol by the Institutional Animal Care Committee of Maastricht University.

LPS-induced acute lung inflammation mouse model
Mice were treated with LPS (Escherichia coli, serotype O55:B5; Sigma, St Louis, MO, USA) by intratracheal (i.t.) instillation to induce an acute pulmonary inflammation. The dose of LPS was 20 μg per instillation per
mouse. No signs of overall toxic effects with the employed dose of LPS were histopathologically observed in the trachea, airways and lungs, either in this study or those executed by others (12). Sham mice were instilled with sterile 0.9% NaCl.

Since our previous study (13) demonstrated that local LPS challenge in Wt mice resulted in a transient neutrophil accumulation peaking at Day 3, Wt mice (n = 6) were sacrificed 3 days post-exposure by 115 mg/kg sodium pentobarbital (Ceva Sante Animale, Maasluis, The Netherlands). After thoracotomy, the lungs were prepared for histological evaluation (as described below) to characterize the LPS-induced inflammatory cell influx. In satellite Wt mice (n = 10), bronchoalveolar lavage (BAL) (5 x with 1 ml sterile 0.9% NaCl) was performed 3 days post-instillation to remove as much as possible inflammatory cells from the airways. After centrifugation at 1500 r.p.m. during 10 min at 4°C, the cell-free bronchoalveolar lavage fluid (BALF) was stored at -80°C for MPO activity measurement. Lavaged lungs were snap frozen and pulverized using a mortar and pestle. The pulverized lung tissue was stored at -80°C for RNA isolation and protein extraction for assessment of NER capacity.

Characterizing the role of MPO, Mpo−/− mice were dosed with LPS, followed by histological evaluation of inflammatory cell influx (n = 6) and BAL (n = 5) with subsequent similar analyses on lung tissue as in Wt animals. Furthermore, to study the direct relation between neutrophils and NER inhibition, depletion of circulating neutrophils in Wt mice (n = 5) was achieved by intraperitoneal injection of 0.5 mg of the monoclonal rat anti-mouse neutrophil antibody NIMP-R14 (Hycult Biotechnology bv, Maastricht, The Netherlands), 24 h before i.t. LPS instillation. NIMP-R14 has been shown to selectively deplete mouse neutrophils in vivo for up to 6 days (14,15). IgG (eBioscience, San Diego, CA, USA) was used as control mAb in the sham group (n = 5). Again, 3 days after instillation, mice were sacrificed, and lungs were lavaged and isolated as described above.

Histopathology and quantification of cellular influx in lung tissue

After thoracotomy, lungs were inflated through the trachea with 10% zinc-buffered formalin (pH 5.5) at a pressure of 20 cm H2O and subsequently placed in 10% zinc-buffered formalin for 24 h for fixation of the tissue. After paraffin embedding, lung sections were cut at a thickness of 4 μm and mounted on slides. Immunohistochemistry and co-stained sections were used to quantify infiltration of neutrophils and macrophages into the lungs. At a magnification of 200×, random fields were selected and the number of infiltrated still intact cells was counted. At least five fields per section were analysed.

MPO activity measurement

MPO packaged in neutrophils will have no effect on NER in pulmonary epithelial tissue because MPO must be released extracellularly during the oxidative burst of neutrophils. Therefore, extracellular MPO activity was measured in cell-free BALF, as described by Klebanoff et al. (16), and is indicative for the presence of activated neutrophils in the lung (17).

Protein extraction

The preparation of lung tissue protein extracts for NER capacity measurement and western blot analysis is based on the method developed by Redaelli et al. (18). Pulverized frozen lung tissue was resuspended in lysis buffer (45 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 0.4 mM KCI, 1 mM ethylenediaminetetraacetic acid, 0.1 mM dithiothreitol and 10% glycerol, adjusted to pH 7.8 using KOH), snap frozen and thawed again to complete lysis and homogenization of the tissue. The lysate was centrifuged at 11,000 x g total RNA. Aliquots were used for total RNA isolation and protein extraction for assessment of NER capacity.

Measurement of NER capacity

To phenotypically assess the NER capacity in lung tissue, we applied a modified comet assay as originally developed and validated in our laboratory (19). This assay measures the ability of NER-related enzymes, present in the prepared lung tissue extracts (250 μg/ml), to detect and incise benzo[a]pyrene-diol epoxide–DNA adducts in substrate DNA. The increase in DNA breaks, leading to increased tail intensities, is indicative for the NER capacity of the tissue extracts. After subtracting background levels from all data, the final repair capacity was calculated as previously reported by Langie et al. (19).

NER gene expression analysis by quantitative polymerase chain reaction

Total RNA was isolated and purified from snap frozen pulverized lung tissue (same tissue that was used for the NER capacity measurement), using the RNAeasy® Mini Kit (Qiagen Westburg, Leusden, The Netherlands) in combination with a DNase treatment, according to the manufacturer’s instructions. First-strand cDNA was generated, using the iScript kit protocol (BIO-RAD) on 1 μg total RNA. Aliquots were used for quantitative polymerase chain reaction on the BioRad MyIQ (Cyler Single Colour quantitative detection system, using iQ SYBR Green Supermix. Reactions were initiated for 3 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 45 sec at 60°C. After each run, a melting curve was analysed starting at 60°C with stepwise temperature elevations of 0.5°C every 10 sec to check for non-specific products. The following NER genes were tested: Xpa, Xpc, Ercc-1, Xpf and Xpg, using primers purchased from Operon (Leiden, The Netherlands) (Table I for primer sequences). Data were analysed, using the MyIQ Software system (BIO-RAD), and were expressed as relative gene expression, using the 2 ΔΔCT method and employing Hprt as housekeeping gene (20).

Determination of XPA levels by western blot analysis

XPA protein abundance was evaluated in the lung protein extracts (that were also used for the NER capacity measurement) by western blotting. Samples were diluted 1:4 in Laemmli sample buffer and boiled for 5 min at 95°C. Equal amounts of protein (40 μl per well) were loaded and separated on a polyacrylamide gel (12%) [Mini-PROTEAN 3 System; BIO-RAD). Subsequently, proteins were transferred to Hybond-C-membrane (Amersham Pharmacia Biotech, Diegem, Belgium) and blocked for 1 h with 5% skim milk powder in phosphate-buffered saline (PBS)0.1% Tween-20. Nitrocellulose blots were washed (PBS0.1% Tween-20), followed by overnight incubation with the polyclonal anti-XPA antibody [XPA (FL-273); sc-853, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA]. After washing, blots were incubated for 1 h with a goat anti-rabbit horseradish peroxidase-conjugated IgG (Dako Cytomation, Glostrup, Denmark) and visualized by means of enhanced chemiluminescence (Amersham Pharmacia Biotech with the LAS-3000 (Fuji). Band intensity was determined using a GS700 densitometer and MOLECULAR ANALYST software (BIO-RAD) and given as mean ± standard error of the mean (SEM). The expected size of the band is 40 kDa, which was further identified by using protein samples from mice that do not express the XPA protein (XPA−/− mice; a generous gift from the Laboratory for Health Protection Research, National Institute for Public Health and the Environment, Bilthoven, The Netherlands), which was analysed together with the samples. A reference sample was included in every blot to correct blot to blot variation.

Statistical analysis

Data are expressed as mean ± SEM and statistical analysis was conducted using the independent samples T-test. Values of P ≤0.05 were taken to denote statistical significance.

Results

LPS-induced acute lung inflammation

To characterize the LPS-induced acute lung inflammation, infiltration of inflammatory cells was histologically quantified in the lung tissue of Wt mice, which revealed that LPS challenge resulted in a massive recruitment of neutrophils in the alveolar spaces (650 ± 190 mm2). Furthermore, LPS instillation resulted in an increase of infiltrating macrophages (40 ± 10 mm2). In the sham-treated group, neither significant neutrophil nor macrophage influx was observed. To verify neutrophil activation in Wt animals, extracellular pulmonary MPO activity was determined in BALF of all mice. In

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agreement with the histological assessment of neutrophil infiltration, a significant MPO activity after LPS instillation in Wt animals was observed (81.4 ± 20.5 mU/ml), whereas no MPO activity was detected in the sham group.

Also in Mpo−/− mice, neutrophil (60 ± 10/mm²) and macrophage (10 ± 1/mm²) numbers were assessed, showing unexpected lower neutrophil, but comparable macrophage infiltration as in Wt mice. The absence of MPO activity in the BALF confirmed the Mpo−/− status. As MPO activity is indicative for the presence of activated neutrophils in the lung (17), MPO was also assessed in BALF of neutrophil-depleted mice, and no MPO activity was detected.

**Intratracheal LPS instillation results in pulmonary NER inhibition**

To reveal whether our previous *in vitro* observation, showing MPO/neutrophil-dependent NER inhibition in co-cultured epithelial cells (10), corresponds with the *in vivo* situation, we examined the role of neutrophils on NER of pulmonary tissue in mice after i.t. LPS instillation using an MPO knockout approach and a neutrophil depletion model. As shown in Figure 1, LPS treatment indeed decreased the pulmonary NER capacity in Wt mice ~2-fold (*P* = 0.05). In contrast to our previous *in vitro* experiments, however, we found that lack of MPO or depletion of neutrophils did not result in a significant abrogation of the pulmonary NER inhibition induced by LPS instillation (Figure 1).

**Intratracheal LPS exposure decreases expression of NER-associated genes**

To address whether the observed LPS-induced NER inhibition in the mouse lung was mediated by a reduction of the corresponding mRNA, we examined pulmonary mRNA expression of NER genes after local LPS exposure *in vivo*. We focused on those enzymes of the NER machinery that are crucially involved in the recognition and incision phase of the NER process because the phenotypical NER assay described above reflects these processes; XPA and XPC for damage recognition, ERCC-1 and XPF as 5’endonucleases and XPG as 3’endonuclease (21).

As shown in Figure 2, almost all the genes exhibited a reduction in expression on LPS exposure to a very similar degree (~50–75%), irrespective of genetic background. However, the regulation of Xpa and Xpf seemed to be neutrophil dependent as these genes were not down-regulated in the LPS-exposed neutrophil-depleted mice. This shift in NER gene down-regulation in the LPS-exposed neutrophil-depleted mice did, however, not result in a different phenotypical effect.

**LPS-induced down-regulation of XPA protein**

XPA protein is the core factor for the assembly of the NER complex and is the only factor in which disruption completely obliterates NER (21,22). Since Xpa gene expression was found to be neutrophil dependently down-regulated after LPS exposure in Wt mice, we sought to determine whether this also occurs at the protein level. Quantification of band intensities indicated differences (~50% decrease, *P* = 0.09) in XPA protein levels between LPS-instilled Wt mice and sham Wt mice but not between LPS-treated Mpo−/− mice compared to their respective sham group (see Figure 3). These data resemble the transcriptional effect of LPS instillation on XPA expression.

**Discussion**

Chronic inflammatory diseases of the lung are associated with lung cancer development. Over the past years, evidence has accumulated that the influx of innate immune cells, including PMN, into the airways contributes to inflammation-associated carcinogenesis (7,8). Our study demonstrated an association between LPS-induced pulmonary inflammation and inhibition of NER in the mouse lung *in vivo*. Considering the exposure to inhaled carcinogens, as well as the simultaneous presence of inflammatory cells in the inflamed lung of smokers for example, we propose that this inflammation-associated reduction of NER may represent a significant and previously unrecognized contributory factor in the development of inflammation-related pulmonary cancer.

Neutrophilia plays a prominent role in host defence against pathogens but is also responsible for pulmonary injury due to the release of several toxic compounds (e.g. proteases), ROS and the enzyme MPO. MPO consumes up to 40–70% of neutrophil-derived H₂O₂ to generate HOCl (23). HOCl is a relative stable and membrane-diffusible molecule that can pass through subcellular compartments and, presumably, can...
reach the cell nucleus (24). Therefore, it has the potential to interact with many different cellular proteins, including DNA repair proteins. In fact, we have previously shown that activated neutrophils are potent inhibitors of the NER pathway in pulmonary epithelial cells via the MPO-catalysed formation of HOCl in vitro (10).

To investigate the association between neutrophil infiltration and NER in the lung in vivo, we applied a mouse model of LPS-induced acute pulmonary neutrophil influx in the airways (12), expanded with an MPO knockout approach for studying the contribution of MPO, and a systemic depletion of neutrophils to reveal the role of neutrophils. We observed in a previous study that i.t. administration of LPS in Wt mice induces lung neutrophilia and associated MPO activation that peaks 3 days after instillation (13). However, a lack of MPO (as in Mpo−/− mice) significantly attenuated the acute inflammatory response induced by airway administration of LPS. This decreased neutrophilia can predominantly be assigned to a decreased extravasation of Mpo−/− neutrophils, as MPO plays a role in the activation of neutrophil adherence before extravasation (25), with a decreased chemokine production as a result (13).

The present in vivo results show that i.t. LPS instillation indeed induces a pulmonary suppression of NER capacity, similar as has been shown in our previous in vitro study although in lesser extent (10). In this in vivo study whole lung tissue homogenates were used, which represent more cell types additional to the epithelial type II cells. This may affect the observed differences in the role of HOCl in the inhibition of NER capacity compared to the in vitro situation. Furthermore, in contrast to the in vitro findings, we were not able to show a distinct role of either MPO or neutrophils in the present in vivo study as LPS instillation in Mpo−/− and neutrophil-depleted animals caused a similar decrease in NER. Although data obtained in the Mpo−/− animals cannot directly be compared with the Wt animals because of the unexpected lower neutrophil influx, the observed NER-inhibiting effect in Mpo−/− mice is nevertheless in line with the depletion model, indicating that lower MPO and a lower number of neutrophils are not affecting LPS-induced NER inhibition.

As a possible explanation of the observed LPS-induced NER inhibition, we addressed the effect of LPS-associated lung inflammation on the transcriptional level of relevant NER genes. Our data showed a suppression of several NER genes in lung tissue after LPS-induced acute lung inflammation, mainly Xpa, Xpc and Xpf in Wt mice. Since XPC is the earliest distortion recognition factor (21), our data imply that LPS instillation affects the ability of lung cells to recognize damaged DNA, which corresponds with our in vitro observations (10). However, there was no clear trend suggestive of

Fig. 2. Relative gene expression profiles of (a) Xpa, (b) Xpc, (c) Xpf, (d) Xpg and (e) Ercc-1 in lung tissue at 3 days after i.t. LPS exposure. Data are presented as mean ± SEM for the LPS-treated Wt mice (n = 8) and Mpo−/− mice and neutrophil-depleted mice (n = 5 per group). Gene expression in the respective sham groups is represented as a 100% line. *P ≤ 0.05 versus sham mice.
Lung inflammation reduces NER in vivo

Mice were exposed to LPS for 3 days after exposure. XPA protein levels were analysed using western blots. (a) Each group represents mean ± SEM (n = 5 mice per group). P = 0.09 versus sham mice. (b) Blots of representative animals for each group are shown.

Fig. 3. Western blot analysis of XPA protein in the lung after LPS instillation. Proteins were extracted from lung tissue of sham and LPS-exposed WT and Mpo−/− mice, 3 days after exposure. XPA protein levels were analysed using western blots. (a) Each group represents mean ± SEM (n = 5 mice per group). P = 0.09 versus sham mice. (b) Blots of representative animals for each group are shown.

The role of neutrophils in the observed NER inhibition may be due to the presence of resident alveolar macrophages in the in vivo situation. Alveolar macrophages are also involved in the lung inflammatory response, more specifically by neutralizing pathogens and recruiting neutrophils in the initiation phase, and apoptotic/cell debris cleanup at a later stage. Additionally, LPS-activated alveolar macrophages generate large amounts of reactive nitrogen species (by activation of inducible nitric oxide synthase), which are originally released for bacterial cell killing but are known to be involved in DNA repair inhibition (29) and may explain the observed neutrophil-independent NER-inhibiting effect of lung inflammation.

In conclusion, our study is the first to show that LPS-induced acute pulmonary inflammation is associated with reduced NER in the mouse lung. This effect appears not to be mediated by the infiltration of neutrophils and the associated release of the enzyme MPO. Nevertheless, these data may provide a further mechanism underlying the association between pulmonary inflammation and lung carcinogenesis.

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