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Published in:
Mutagenesis

DOI:
10.1093/mutage/gep049

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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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 (3–10-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). Intratracheal instillation was performed by a non-surgical technique under anaesthesia as previously described (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 neutrophil accumulation peaking at Day 3, Wt mice (n = 6) were sacrificed 3 days post-exposure by 115 mg/kg sodium pentobarbitol (Ceva Sante Animale, Maassluis, The Netherlands). After thoracotomy, the lungs were prepared for histological examination (as described below) to characterize the LPS-induced inflammatory cell influx. In satellite Wt mice (n = 10), bronchoalveolar lavage (BAL) (3× 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.

To characterize the role of MPO, Mpo−/− mice were dosed with LPS, followed by histological examination 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 during 24 h for fixation of the tissue. After paraffin embedding, lung sections were cut at a thickness of 4 μm and mounted on slides. De-paraffinized and eosin-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 KCl, 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. Linebro tissue extracts for NER capacity measurement) by western blotting. Samples were diluted 1:4 in Laemmli sample buffer and boiled for 5 min at 95°C. Bands were visualized by means of enhanced chemiluminescence (Amersham Pharmacia Biotech) with the LAS-3000 (Fuji). Band intensity was determined by using a GS700 densitometer and MOLECULAR ANALYST software (BIO-RAD) and given as mean ± standard deviation (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 for blots 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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 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, Xpg and Xpg, usingprimers purchased from Operon (Leiden, The Netherlands) (see 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).
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

![Fig. 1. Effect of LPS-induced neutrophil influx in the lung on phenotypical NER capacity of lung cells. Phenotypical NER capacity was determined using protein extracts from the lavaged lungs of Wt, $Mpo^{-/-}$ and neutrophil-depleted mice, 3 days after LPS instillation. Data are expressed as mean ± SEM of —four to nine mice per group. $*P \leq 0.05$ versus sham mice.](https://academic.oup.com/mutage/article-abstract/25/1/77/1356356)
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 \textit{in vitro} (10).

To investigate the association between neutrophil infiltration and NER in the lung \textit{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\textsuperscript{-/-} 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\textsuperscript{-/-} 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 \textit{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 \textit{in vitro} study although in lesser extent (10). In this \textit{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 \textit{in vitro} situation. Furthermore, in contrast to the \textit{in vitro} findings, we were not able to show a distinct role of either MPO or neutrophils in the present \textit{in vivo} study as LPS instillation in Mpo\textsuperscript{-/-} and neutrophil-depleted animals caused a similar decrease in NER. Although data obtained in the Mpo\textsuperscript{-/-} animals cannot directly be compared with the Wt animals because of the unexpected lower neutrophil influx, the observed NER-inhibiting effect in Mpo\textsuperscript{-/-} 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 \textit{in vitro} observations (10). However, there was no clear trend suggestive of

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**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\textsuperscript{-/-} 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.
differences in the way different types of mice respond to LPS instillation, except for the neutrophil-mediated down-regulation of Xpa and Xpf. XPA is the only crucial core factor in the NER complex configuration showing affinity for damaged DNA and key interactions with the other NER enzymes, which disruption completely obliterates NER (22). From this point of view, XPA seems to be a neutrophil-specific target for inflammation-associated NER disruption by down-regulating and possibly inhibiting its function. Although Xpa was not down-regulated in Mpo−/− mice and neutrophil-depleted animals, the down-regulation of the other relevant NER-related genes may be responsible for the decreased phenotypical NER capacity seen in these mouse models, as probably all XP proteins of the NER repair complex are needed for effective DNA repair. Interestingly, the in vivo finding on Xpa down-regulation was corroborated by decreased XPA protein in the inflamed lungs, further explaining the distortion of the NER process seen in the LPS-exposed Wt animals. Referring to our previous in vitro observations (10), this reduced XPA protein expression implicates also a role for HOCl-mediated oxidative inactivation of repair enzymes or other HOCl–NER protein interactions in neutrophil-induced NER inhibition, although additional studies are needed to assess this.

A clarification for the differing findings on MPO dependency in vivo and in vitro can possibly be found in a study of Aratani et al. (26), comparing Mpo−/−, Nadph oxidase−/− and double (Mpo/Nadph−/−) knockout mice in a model of pulmonary infection. They reported that H2O2 used by MPO is solely produced by reduced nicotinamide adenine dinucleotide phosphate oxidase. It is therefore likely that Mpo−/− mice that are incapable of inverting H2O2 to HOCl build up H2O2. Since H2O2 is a very potent oxidant, just like HOCl, increased H2O2 levels in the Mpo−/− mice could exert some NER-suppressive effects by oxidative modulation of NER-related genes (especially Ercc-1 expressional down-regulation) on the lung target cells, which is in line with studies from our own laboratory (27) and Hu et al. (28).

Moreover, the in vivo versus in vitro discrepancy with respect of 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.

**Funding**

Province of Limburg, The Netherlands; European Network of Excellence 'Environmental cancer, nutrition and individual susceptibility', sixth Framework programme, FOOD-CT-2005-513943.

**Acknowledgements**

We thank Yonca Güngör for linguistically reviewing the manuscript. The authors thank Dr Aratani Y. for the Mpo−/− mice.

Conflict of interest statement: None declared.

**References**


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**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 column represent mean ± SEM (n = 5 mice per group). P = 0.09 versus sham mice. (b) Blots of representative animals for each group are shown.


Received on July 14, 2009; revised on October 7, 2009; accepted on October 7, 2009.