The role of macrophage mediators in respirable quartz-elicited inflammation

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Abstract. The instigation and persistence of an inflammatory response is widely considered to be critically important in quartz-induced lung cancer and fibrosis. Macrophages have been long recognised as a crucial player in pulmonary inflammation, but evidence for the role of type II epithelial cells is accumulating. Investigations were performed in the rat lung type II cell line RLE and the rat alveolar macrophage cell line NR8383 using Western blotting, NF-κB immunohistochemistry and qRT-PCR of the pro-inflammatory genes iNOS and COX-2, as well as the cellular stress gene HO-1. The direct effect of quartz on pro-inflammatory signalling cascades and gene expression in RLE cells was compared to the effect of conditioned media derived from quartz-treated NR8383 cells. Conditioned media activated the NF-κB signalling pathway and induced a far stronger upregulation of iNOS mRNA than quartz itself. Quartz elicited a stronger, progressive induction of COX-2 and HO-1 mRNA. Our results suggest a differentially mediated inflammatory response, in which reactive particles themselves induce oxidative stress and activation of COX-2, while mediators released from particle-activated macrophages trigger NF-κB activation and iNOS expression in type II cells.

1. Introduction
Exposure to high concentrations of respirable crystalline silica is well known to cause progressive fibrosis and lung cancer in rats. In humans, respirable quartz induces silicosis, a disease that is associated with an increased risk of lung cancer [1]. Based on chronic inhalation studies in the rat and epidemiological data, the ability of quartz to elicit a marked and persistent inflammation is believed to be a crucial factor for the development of these severe pathogenic conditions [2, 3]. The cell types orchestrating the initial inflammatory response after particle exposure are thought to be the alveolar macrophage and epithelial cells, two main airway cell types likely to interact with inhaled particles; however, the contribution of each is not fully known and the mechanisms involved in their activation are at present not clearly understood. On a cellular level, the central regulator activating cells in response to quartz is considered to be transcription factor nuclear factor κB (NF-κB) [4]. In its dormant state, NF-κB, typically present as a dimer of the RelA (p65) and p50 subunits, is bound in the cytosol to its inhibitor protein, which is usually IκBα. In the classical NF-κB activation pathway, an
NF-κB activating stimulus reaches the cell, upon which the inhibitor protein IkBα is phosphorylated at serines 32 and 36, ubiquitinated at lysines 21 and 22, and subsequently degraded by the 26S proteasome. This liberates the NF-κB unit from the nuclear export signal of IkBα, and allows for its migration into the nucleus where it activates the transcription of many pro-inflammatory genes [4, 5].

Previously, we and others have shown that crystalline silica can activate the NF-κB pathway in vivo [6, 7, 8]. When considered together, these studies are indicative of NF-κB activation within alveolar macrophages, but also in bronchiolar as well as alveolar epithelial cells. Macrophages have been long recognised as a crucial player in pulmonary inflammation, but evidence for the role of epithelial cells is accumulating [9]. Pro-inflammatory activation of lung epithelial cells in quartz-exposed lungs principally may involve two principal pathways, i.e. (1) direct activation of the epithelium by quartz particles or (2) activation of the epithelium by mediators released from quartz-interacting macrophages. Recently we provided further support for such an indirect mechanism by showing that bronchoalveolar lavage fluids from quartz-exposed rats can trigger NF-κB activation in lung epithelial cells in vitro [8]. The present work was undertaken to further investigate the role of macrophages in quartz-induced inflammation, focusing on quartz vs. macrophage mediated activation of the NF-κB pathway in lung epithelial cells. Moreover, we evaluated the mRNA expression of inducible Nitric Oxide Synthase (iNOS) and cyclooxygenase-2 (COX-2) because of their discussed involvement in quartz-induced lung disease [6, 10, 11, 12, 13], as well as of heme oxygenase-1 (HO-1) which is considered a sensitive marker of particle-induced oxidative stress [13, 14].

2. Methods

For the present study, two cell types were used, i.e. the rat alveolar macrophage cell line NR8383 [15] and the rat lung epithelial cell line RLE-6TN (RLE) [16]. Dörentruper quartz (DQ12, batch 6 IUF, mean diameter of 0.96μm) was used as a standard respirable quartz sample. In order to inactivate any endotoxins that might be present at the particle surface, DQ12 was baked at 220°C for 24h. For cell treatments, DQ12 was suspended in reduced RLE medium (Ham’s F12 containing 0.1% FCS, 1% γ-glutamine, 1% penicillin and 1% streptomycin) or normal NR8383 medium (F12-K with 15%FCS, 1% γ-glutamine, 1% penicillin and 1% streptomycin), respectively. Particle suspensions were prepared immediately prior to treatment and added to the cells after sonicated for 5 minutes in a water-bath sonicator (60Watt, 35Hz, Sonorex TK 52, Schaltech, Mörfelden-Walldorf, Germany). The experimental approach of the study is shown in Figure 1.

2.1. Culture and treatment of NR8383 cells and RLE cells

NR8383 macrophages were seeded in 60-mm culture dishes at a concentration of 0.5 × 10^6/ml and then cultivated for 3 days at 37°C and 5% CO until they reached 60% confluence. Subsequently, cells were treated with DQ12 at concentrations and time intervals as indicated in the results section. Control cells received fresh NR8383 medium. After incubation, supernatants were harvested for treatment of RLE cells. Obtained supernatants destined for RLE treatment were spun at low speed (200g for 5 min) to remove non-adherent cells, and spun once more at 18000g for 5 min to remove cell debris and particles. RLE cells were seeded in 60mm or 100mm culture dishes at a concentration of 3 × 10^4 cells/cm^2 and cultivated in respectively 2 or 6ml complete Ham’s F12 medium. At 90% confluence, medium was replaced by reduced medium containing 0.1% FCS for 24h for cell synchronization. Cells were incubated for various time intervals with DQ12 particles at a concentration of 40μg/cm^2 (direct treatment), a concentration selected on the basis of prior concentration range finding experiments. To compare this direct treatment with the indirect, macrophage-mediated induction, cells were treated with 1:6 diluted supernatants of untreated or DQ12-treated NR838 cells. Recombinant rat Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β), as well as TNF-α and IL-1β neutralizing antibodies (R&D Systems, Germany) were used to determine the role of the inflammatory cytokines Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) on NF-κB pathway activation by DQ12 conditioned macrophage media.
Figure 1. Experimental approach: RLE cells were exposed either to DQ12 quartz (A) or to the conditioned medium obtained from untreated or quartz treated NR8383 cells (B). For both models, the RLE cells were then evaluated for NF-κB activation by evaluation of phosphorylation and degradation of its cytosolic inhibitor IκBα and translocation of its RelA (p65) subunit, as well as for mRNA expression of iNOS, COX-2 and HO-1.

2.2. Immunocytochemistry
RLE cells were cultured to near confluence on 4-chamber culture slides (BD Falcon, Heidelberg Germany). After treatment, cells were rinsed twice with PBS and fixed in 4% paraformaldehyde/PBS (pH 7.4) followed by endogenous peroxidase inactivation with 0.3% H2O2 for 30 min. After permeabilisation in citrate buffer (pH 6.0) by microwave treatment, slides were blocked with normal goat serum (1:65) for 1 h to prevent nonspecific binding. Incubation with a rabbit polyclonal antibody against NF-κB RelA (Santa Cruz Biotechnology, USA) was followed by a secondary goat anti-rabbit fluorescent antibody (MFP555, 1:200, MoBiTec, Göttingen, Germany) and nuclear counterstaining with Hoechst 33342 (1µg/ml Sigma, Deisenhofen, Germany). After dehydration slides were covered in DePex (Serva, Heidelberg, Germany). As a negative control, slides of each treatment were incubated with rabbit IgG instead of the primary antibody at the same IgG concentration. Slides were analyzed using a fluorescence microscope (Olympus BX60) at an original magnification of ×1000.

2.3. Western blotting
RLE cells were harvested by gentle scraping and incubated for 30 min in 200µl lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in PBS) at 4°C containing freshly added protease inhibitors (Complete cocktail; Roche, Penzberg, Germany). Subsequently, lysates were centrifuged for 20min at 15000g and 4° C. Protein concentrations were determined using the bicinchoninic acid (BCA) assay and Equal amounts (20µg) were electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto nitrocellulose membranes. Non-specific protein binding was blocked with 5% dried milk powder and 0.1% Tween-20 in PBS. IκBα protein was detected using a rabbit polyclonal antibody (1:1000, C-21, Santa Cruz Biotechnology) and an anti-rabbit-IgG whole protein HRP-conjugated secondary antibody (1:3000, Sigma). To detect IκBα phosphorylated at serine 32 and/or 36, a monoclonal antibody was used (1:2000) (ser32/36, clone 16A6, Cell Signaling Technologies, Danvers USA) in combination with an anti-mouse-IgG
whole protein HRP-conjugated secondary antibody (1:2000, Sigma). Band formation was visualized using an ECL Plus reagent/detection system (Amersham Bioscience Europe, Freiberg, Germany).

2.4. Quantitative rtPCR analysis of gene expression in RLE cells

RLE cells were transferred into Trizol (Invitrogen, Karlsruhe Germany). Subsequently RNA was extracted according to the manufacturer's instructions. The RNeasy® mini kit coupled to DNase treatment (RNase-free DNase set, Qiagen) was used to purify total RNA from salts and residual DNA. Quantity and purity of RNA were evaluated using spectrophotometry. cDNA was synthesized using the iScript cDNA Synthesis kit (BioRad, CA, USA), starting from 0.5 µg of RNA. cDNA was diluted 15x in water before use. rtPCR primers for rat HO-1, iNOS, COX-2 and the housekeeping gene HPRT were designed using Primer express software (Applied Biosystems) and obtained from Operon. Primer sequences for iNOS were 5'-AGG AGA GAG ATC CGG TTC ACA GT-3' (forward) and 5’-ACC TTC CGC ATT AGC ACA GAA-3' (reverse), for COX-2 5’'-GCA CAA ATATGA TGT TCG CAT TCT-3’ (forward) and 5'-GAA CCC AGG TCC TCG CTT CT-3’ (reverse), for HO-1 5’-GGG AAG GCC TGG CTT TTTT -3' (forward) and 5'-CAC GAT AGA GCT GTT TGA ACT TGGT -3' (reverse), and for HPRT 5’-GCC CTG TTT CAC TCA AAT GAG CTG CTT TAT A-3’ (forward) and 5’-TCT TTT AGG CTT TGT ACT TGG CTT TT-3’ (reverse). Real time PCR was performed with a MyiQ Single Colour real time PCR detection system (BioRad) using SYBR© Green Supermix (Biorad, CA, USA), 5 µl diluted cDNA, and 0.3 µM primers in a total volume of 25 µl. PCR was conducted as follows: a denaturation step at 95°C for 3 min was followed by 40 cycles at 95°C (15 seconds) and 60°C (45 seconds). Melt curves (60-95°C) were produced for product identification and purity. PCR efficiency of all three primer sets, as assessed by the use of cDNA dilution curves, was 90-100%. Data were analyzed using the MyiQ Software system (BioRad) and were expressed as relative gene expression (fold increase) using the 2-ΔΔCt method [17].

![Western blots of IκBα degradation and phosphorylation in RLE cells.](image)

**Figure 2.** Western blots of IκBα degradation and phosphorylation in RLE cells. A: Effect of DQ12 on IκBα degradation in RLE cells. Cells were treated with 40µg/cm2 DQ12 for the indicated time intervals. B: Effects of conditioned medium from untreated vs. DQ12-treated NR8383 macrophages on IκBα degradation (B) and IκBα Ser-32/36-phosphorylation. RLE cells were treated for the indicated time intervals with a 1:6 dilution of supernatants from untreated NR8383 cells or NR8383 cells that were treated for 24h with 40 µg/cm² DQ12.
3. Results

To compare NF-κB activation in RLE cells induced by DQ12 particles (direct effect) with that as induced by the supernatants of quartz treated macrophages (indirect, macrophage-mediated effect), we first investigated IκBα phosphorylation and degradation by Western blotting analysis (Figure 2). Direct treatment of RLE cells with quartz, did not result in a significant degradation of IκBα degradation (Fig. 2A). In concordance with these findings no phosphorylation of IκBα could be detected (not shown). In contrast, conditioned media obtained from quartz treated NR8383 cells were found to cause a fast and strong degradation of IκBα (Fig. 2B). The degradation of this NF-κB inhibitor protein was found to be preceded by its phosphorylation (Fig. 2C). The fast phosphorylation and degradation of IκBα was followed by a rapid restoration (30 to 60 min), indicative of its de novo synthesis. In RLE cells that were treated with supernatants from untreated NR8383 cells also a mild IκBα reduction was observed, but in the absence of any detectable phosphorylation (Figs 2B and C).

![Figure 2](image1.png)

**Figure 2.** Immunocytochemical analysis of the nuclear translocation of the NF-κB subunit RelA in RLE lung epithelial cells after treatment with conditioned medium from untreated NR8383 cells (A, B) or conditioned medium from DQ12-treated NR8383 cells (C, D). Cells were treated for 10 minutes with a 1:6 dilution of supernatants obtained from untreated NR8383 cells or from NR8383 cells that were treated for 6 hours with 40μg/cm² DQ12. Staining for RelA is shown in panels A and C. For the verification of its nuclear translocation, counterstaining with Hoechst 33342 was used, shown in panels B and D for the respective treatments.
Supernatants from DQ12 treated macrophages were also found to elicit a marked nuclear translocation of RelA in RLE cells, while supernatants from untreated NR8383 cells failed to cause such clear nuclear translocation (Figure 3). These findings were in concordance with their respective effects on phosphorylation and degradation of IkBα as observed obtained in the Western blot analysis (Figure 2). In RLE cells that were treated directly with DQ12 quartz, RelA nuclear translocation was also absent (not shown). When taken together, the Western blot and immunocytochemical analysis suggest that NF-κB activation induced in RLE cells by quartz, occurs via the indirect mechanism, mediated by macrophage products, rather than directly through the quartz particles themselves. The data also indicate that this NF-κB activation occurs through the classical, IkB-degradation-mediated pathway, involving phosphorylation and degradation of the most common IkB molecule, IkBα.

The potential role of TNFα and/or IL-1β in the indirect, macrophage mediated NF-κB activation we investigated the effects of these cytokines on RLE cells combined with analysis of effects of their inhibitors. As shown in Fig. 4, TNFα was induced a clear IkBα degradation followed by a rapid restoration. However, anti-TNFα neutralizing antibodies failed to block activation of the classical NF-κB pathway in RLE cells as induced by supernatants from DQ12-treated macrophages, while completely blocking the effect of TNFα (Figure 4). Such findings were also obtained for IkBα ser32/36 phosphorylation and RelA nuclear translocation, as well as in further blocking experiments applying IL-1β neutralizing antibody or even a combination of both TNFα and IL-1β inhibitors (not shown). Taken together, these results indicated that the acute inflammatory cytokines TNFα and IL-1β do not play a crucial role in the indirect macrophage-mediated NF-κB activation in RLE cells.

Figure 4. Western blot analyses on the investigation of the involvement of TNFα in indirect, macrophage mediated IkBα degradation in RLE cells. A. Degradation of IkBα degradation in RLE cells upon treatment with TNFα at indicated concentration and time points. B. Expression of IkBα protein in RLE cells after treatment with NR8383 supernatants that were pretreated with a TNFα neutralizing antibody. For these experiments, supernatants from untreated as well as DQ12-treated NR8383 macrophages were incubated for 1h with anti-TNFα at a concentration of 5μg/ml and then added to RLE cells in a 1:6 dilution.

To further investigate and compare the direct versus indirect pro-inflammatory effects of DQ12 quartz on lung epithelial cells, RLE cells were also evaluated on the mRNA expression levels for of iNOS, COX-2 and HO-1 by quantitative rtPCR. The findings of these experiments are summarised in
The supernatants from the DQ12 treated NR8383 macrophages were found to be capable of inducing iNOS almost 2000-fold in the RLE cells, whereas direct DQ12 treatment elicited an approximate 50-fold increase of the same transcript. In contrast, COX-2 mRNA expression was found to be induced more strongly in the RLE cells upon direct treated with DQ12 than upon treatment with the macrophage supernatants. In line with COX-2, also HO-1 mRNA expression was found to be relatively stronger increased in the RLE cells upon direct quartz treatment (see Table 1).

Table 1. Induction of HO-1, iNOS and COX-2 mRNA expressions, in RLE lung epithelial cells after treatment with treatment with DQ12 quartz versus DQ12-treated NR8383 macrophages.

|                      | iNOS (fold induction) | COX-2 (fold induction) | HO-1 (fold induction) |
|----------------------|-----------------------|------------------------|-----------------------|
| Treatment with       | 1845.8 ± 1038.9        | 12.9 ± 8.1              | 2.7 ± 1.7             |
| conditioned media    | (2h)                  | (2h)                   | (2h)                  |
| from DQ12-treated MΦ |                       |                        |                       |
| Treatment with DQ12  | 55.0 ± 12.4            | 164.9 ± 10.5            | 15.7 ± 4.5            |
| (8h)                 |                       | (4h)                   | (8h)                  |
| ratio of fold induction | 33                     | 0.08                   | 0.17                 |
| (MΦ supernatants vs. DQ12) |                      |                        |                       |

The fold induction compared to untreated cells is shown upon treatment of RLE cells with DQ12 for 4 or 8 hours with supernatants from DQ12-treated NR8383 cells for 2 hours. The selected time points were those showing the highest induction (indicated between brackets) of the respective genes as determined from the kinetic investigations (2 - 8 hours).

4. Discussion and conclusion

In this study we found that conditioned media from quartz-treated macrophages activated the NF-κB signalling pathway and induced a far stronger upregulation of iNOS mRNA than quartz itself in epithelial cells. In contrast, quartz particles elicited a stronger, progressive induction of COX-2 and HO-1 mRNA, in the absence of any notable NF-κB activation. The gene encoding iNOS is known to be NF-κB regulated [18], and has been implicated in silica induced inflammation and fibrosis in the basis of studies with iNOS knockout mice [10, 11]. Our current in vitro findings show that the effect of macrophage products secreted in response to quartz on NF-κB activation in alveolar epithelial cells is greater than the effect of the quartz particles directly, providing further support for the macrophage as crucial mediator of (quartz-induced) inflammation [9, 19, 20]. Our findings also showed that TNFα and IL-1β do not play a crucial role in quartz-induced NF-κB activation in RLE cells, but that other factors are at least involved or possibly more important.

The observed induction of COX-2 mRNA expression by DQ12, however, also suggests a role for direct particle-epithelial interactions in triggering pro-inflammatory effects. COX-2 is involved in the pathogenesis of various inflammatory lung diseases [21]. Although COX-2 is known to be regulated by NF-κB [22], in the present study DQ12 treatment did not lead to any notable induction of this pathway in the RLE cells. Since this treatment also induced the mRNA expression of the oxidative stress marker HO-1, it appears that quartz particles induce COX-2 expression via the activation of NF-κB independent, yet oxidative stress-dependent, cell signalling. Finally, the observed profiles of HO-1 mRNA expression in our study is also of interest for two further reasons. On the one hand, the failure of macrophage mediators to strongly activate this gene indicates that the indirect (macrophage mediated) epithelial NF-κB activation occurs rather in an oxidative stress-independent manner. On the
other hand, the ability of quartz to directly activate HO-1 in epithelial cells provides further support for direct quartz-driven pro-inflammatory effects, as silica-induced inflammation in mice was recently shown to depend on HO-1 expression in the lung [23]. In conclusion, our results suggest a differentially mediated inflammatory response, in which reactive particles themselves induce oxidative stress and activation of COX-2, while mediators released from particle-activated macrophages trigger NF-κB activation and iNOS expression in type II cells.

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