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# Curcumin regulates airway epithelial cell cytokine responses to the pollutant cadmium

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ABSTRACT

Cadmium is a toxic metal present in the environment and its inhalation can lead to pulmonary disease such as lung cancer and chronic obstructive pulmonary disease. These lung diseases are characterized by chronic inflammation. Here we show that exposure of human airway epithelial cells to cadmium promotes a polarized apical secretion of IL-6 and IL-8, two pivotal pro-inflammatory cytokines known to play an important role in pulmonary inflammation. We also determined that two distinct pathways controlled secretion of these proinflammatory cytokines by human airway epithelial cells as cadmium-induced IL-6 secretion occurs via an NF-κB dependent pathway, whereas IL-8 secretion involves the Erk1/2 signaling pathway. Interestingly, the natural antioxidant curcumin could prevent both cadmium-induced IL-6 and IL-8 secretion by human airway epithelial cells. In conclusion, curcumin could be used to prevent airway inflammation due to cadmium inhalation.



## 1. Introduction

Cadmium is an environmental pollutant that is currently ranked 7th on the Priority list of Hazardous Substances by the Agency for Toxic Substances and Disease Registry (ATSDR). While only 5–10% of ingested cadmium is absorbed systemically, pulmonary absorption of inhaled cadmium can reach 80% [1,2]. The level of cadmium in the lung of smokers has been shown to be around 30 μM and could be higher in some areas [3]. Cadmium inhalation has been linked to lung cancer and chronic obstructive pulmonary disease (COPD) [4,5]. These diseases are characterized by chronic inflammation; cadmium has recently been shown to induce inflammation in the lung of mice [6].

In the lung, airway epithelial cells are the first line of defense and in addition to being a physical barrier, the epithelium can respond to pollutants by secreting inflammatory mediators and by recruiting immune cells [7,8]. Cadmium was recently shown to induce secretion of interleukin-6 (IL-6) and IL-8 in primary cell cultures from rat lungs [9]. These cytokines have been found to be elevated in mouse models and humans with chronic lung disease [10–12]. IL-6 secretion in the lung is associated with lung injury [13]. On the other hand, IL-8 is a chemoattractant cytokine responsible for the recruitment of neutrophils and macrophages into the sites of inflammation. Prolonged secretion of IL-8 can have deleterious effects on the lung due to release of toxic products (such as reactive oxygen intermediates) by accumulated neutrophils and macrophages [14–16].

Curcumin (diferuloylmethane) is a naturally occurring polyphenolic pigment from the Indian spice turmeric that is isolated from the rhizomes of the plant *Curcuma longa* Linn. Curcumin has antitumor, antioxidant, and anti-inflammatory properties. It has long been used in ancient medicine in particular in India and China. It is now part of several clinical trials, mainly for cancer therapy, for its ability to induce apoptosis in cancer cells [17,18]. The anticancer and anti-inflammatory properties of curcumin are in part due to inhibition of the nuclear factor kappa B (NF-κB) pathway [19,20]. In spite of this, little is known about the anti-inflammatory mechanism of curcumin in the lung.

Several studies showed that high doses of cadmium induce toxicity and cell death, whereas low doses induce inflammation. Here we examined the effect of subtoxic concentrations of cadmium on secretion of cytokines by human airway epithelial cells and the effect of curcumin. We found that curcumin could prevent secretion of both IL-6 and IL-8. We also show that curcumin inhibits the secretion of IL-8 induced by cadmium by preventing activation of the MEK/Erk1/2 pathway.

## 2. Materials and methods

### 2.1. Tissue culture and reagents

The human bronchial epithelial (HBE) cell line Calu-3 was cultured as previously described [21]. Cadmium sulfate (Sigma, St.

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Louis, MO) was dissolved in sterile water at a concentration of 100 mM and was diluted in cell culture media to reach the indicated concentration. MAPK inhibitor UO126 was from Calbiochem (La Jolla, CA), and the NF-κB inhibitor Bay 11-7082 was from Santa Cruz Biotechnology (Santa Cruz, CA).

### 2.2. ELISA for analysis of cytokines released into cell supernatants

Confluent HBE cells, Calu-3, were treated as previously described [22]. Unless specified otherwise, the experiments were carried out using MAPK inhibitor at a concentration of 10 μM and NF-κB inhibitor Bay 11-7082 at a concentration of 5 μM. CXCL8/IL-8 and IL-6 were quantified by ELISA following the manufacturer's protocol (R&D Systems, Minneapolis, MN). Each data point represents the mean from a minimum of three independent assays performed in triplicate. Data are expressed as pg/ml.

### 2.3. Quantitative RT-PCR (qRT-PCR) analysis

Real-time quantitative RT-PCR was employed to measure the transcript levels of IL-8 and IL-6 as previously described [21]. The IL-8 and IL-6 mRNA levels were normalized to the expression of the housekeeping gene CAP-1. Messenger RNA levels were expressed as relative copy number (RCN), which was calculated using with the following equation: $RCN = 2^{-\Delta Ct} \times 100$ where $\Delta Ct = Ct_{(target)} - Ct_{(housekeeping\ gene)}$.

### 2.4. Measurement of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [21]. Cells were treated with cadmium and/or curcumin for 24 h. At the end of the treatment, the number of viable cells was determined by measuring their capacity to convert a tetrazolium salt into a blue formazan product.

### 2.5. Determination of NADPH oxidase activity by chemiluminescence assay

NAD(P)H oxidase activity in intact cells was assayed by lucigenin chemiluminescence assay according to Parinandi et al. (2003) with a slight modification [23]. Fifty microliters of cell suspension was added to a final 1 ml volume of pre-warmed (37 °C) phenol red-free medium (DMEM) containing either NADPH (50 μM) or lucigenin (20 μM) to initiate the reaction, followed by immediate measurement of chemiluminescence in a Berthold Luminometer. Appropriate blanks and controls were established, and chemiluminescence was recorded. Chemiluminescence was measured continuously for 1 min, and the activity of NAD(P)H oxidase was expressed as relative chemiluminescence units (RLU).

### 2.6. Cell stimulation and immunoblotting

Confluent HBE Calu-3 cells were placed in serum-free media overnight. Cells then were stimulated with cadmium sulfate at the concentration and time indicated. Detection of phospho Erk1/2 was performed as previously described [21]. Each Western blot is representative of at least three independent experiments.

### 2.7. Statistical analysis

Data are expressed as mean ± standard errors (SE) of at least three independent experiments. Statistically significant differences were assessed using Student's *t*-test. *P* values <0.05 were considered significant.

## 3. Results

### 3.1. Cadmium induces polarized secretion of IL-6 and IL-8 by human bronchial epithelial (HBE) cells

Since cadmium inhalation has been associated with lung diseases where inflammation plays a key role, we investigated whether cadmium directly induces secretion of pro-inflammatory cytokines by human airway epithelial cells. For this purpose, we used Calu-3 cells, which represent a good *in vitro* model to study HBE cells including inflammatory responses [24,25]. HBE cells grown on permeable supports are fully polarized and allow collection of both apical and basal media for analysis. Various concentrations of cadmium were added to the apical compartment to mimic cadmium inhalation and cytokines were measured in the apical or basal medium. As observed in Fig. 1A and B, IL-6 and IL-8 were secreted primarily into the apical compartment in a dose-dependent manner. IL-8 could also be detected in the basal compartment under basal (unstimulated) conditions. Cadmium treatment had no effect on IL-8 secretion in this latter compartment (Fig. 1A). Conversely, IL-6 was not detected in the basal compartment even after treatment with 50 μM cadmium (IL-6 <10.3 pg/ml) (Fig. 1B).

### 3.2. Distinct pathways control secretion of IL-8 and IL-6 by HBE cells exposed to cadmium

In order to identify the signaling pathway(s) that control secretion of IL-8 and IL-6 in response to cadmium by human airway epithelial cells, HBE cells were incubated with the NF-κB inhibitor Bay 11-7082, and MEK/Erk1/2 inhibitor UO126. As observed in Fig. 1C and D, inhibition of the NF-κB pathway had no effect on secretion of IL-8 whereas addition of the MEK inhibitor UO126 completely suppressed IL-8 secretion. Conversely, addition of the NF-κB inhibitor Bay 11-7082 significantly decreased the cadmium-induced secretion of IL-6 whereas inhibition of the MEK/Erk1/2 pathway had no effect (Fig. 1E and F). These results indicate that cadmium induces secretion of IL-6 and IL-8 by two distinct signaling pathways in HBE cells.

### 3.3. The antioxidant curcumin prevents secretion of both IL-6 and IL-8 induced by cadmium

Our results show that cadmium induces secretion of IL-6 and IL-8 by human airway epithelial cells via NF-κB-dependent and -independent mechanisms, respectively. NF-κB is a main pathway that controls secretion of inflammatory cytokines. Curcumin was shown to exert its anti-cancer effect through inhibition of the NF-κB pathway [19,20]. Since cadmium induces secretion of IL-8 via an NF-κB-independent mechanism, we investigated whether curcumin could prevent the cadmium-induced secretion of IL-8. As seen in Fig. 2A, curcumin decreased secretion of IL-8 in a dose-dependent manner. In fact, concentrations of curcumin above 30 μM completely blocked the cadmium-induced secretion of IL-8. Since cadmium induces secretion of IL-6 via an NF-κB-dependent mechanism, we expected curcumin to prevent its secretion by HBE cells. Accordingly, we found that curcumin decreases secretion of IL-6 in a dose-dependent manner (Fig. 2B). At a concentration of 10 μM, curcumin slightly decreased secretion of IL-6 and completely suppressed the cadmium-induced secretion of IL-6 when concentrations above 20 μM were used. To confirm that cadmium in combination with curcumin was not toxic to the cells, cell viability was investigated by MTT assay. When compared to control cells, no decrease in cell viability was observed when HBE cells were treated with cadmium (50 μM)

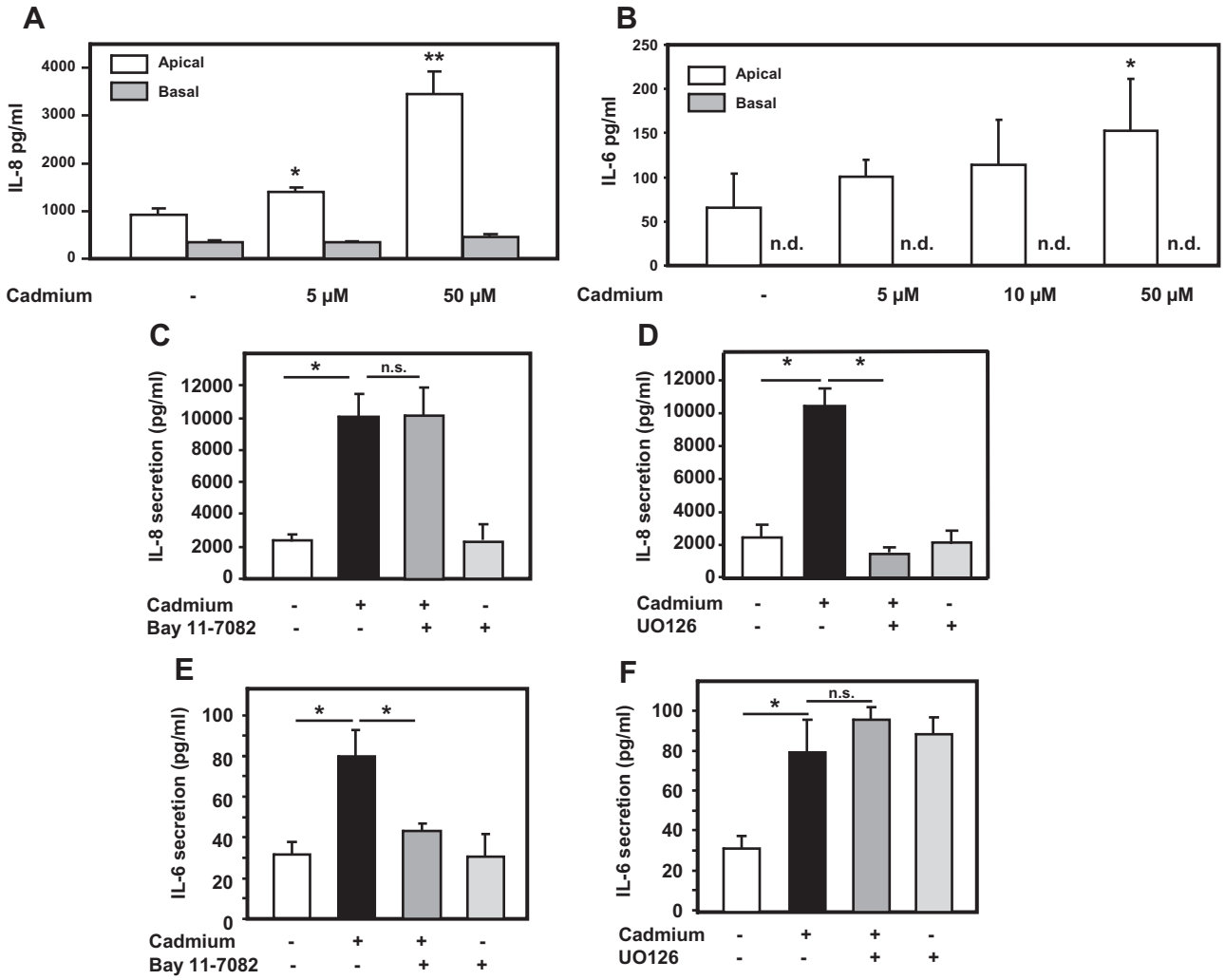


**Fig. 1.** Cadmium induces secretion of IL-8 and IL-6 by human airway epithelial cells. (A and B) Polarized human bronchial epithelial (HBE) cells were treated for 24 h with the indicated concentrations of cadmium. (C–F) HBE cells were treated with 50 μM cadmium for 24 h in the presence of 20 μM UO126 (MEK/Erk1/2 inhibitor) (C and E), or 5 μM Bay 11-7082 (NF-κB) (D and F). IL-8 (A, C, and D) and IL-6 (B, E and F) released in the supernatant were measured by ELISA. "n.d." indicates that the levels were "not detectable". Data are expressed as mean ± SE of at least three independent experiments. $^{*}p < 0.05$; n.s., not significant.

and/or curcumin (30 μM) for 24 h (82 ± 5% of control for cadmium alone and 78 ± 8% of control for cadmium and curcumin). In another set of experiments, we investigated the effect of the antioxidant α-tocopherol, a form of vitamin E, could also suppress secretion of IL-8 and IL-6 in response to cadmium. As seen in Fig. 2C and D, α-tocopherol did not suppress release of IL-8 and IL-6 in supernatants in response to cadmium. These results suggest that not all antioxidants can prevent production of cytokines in response to cadmium.

### 3.4. Curcumin prevents up-regulation of IL-6 and IL-8 mRNA transcript levels induced by cadmium

We next measured mRNA transcript levels to address whether curcumin was affecting IL-6 and IL-8 at the post-transcriptional level. In these studies, we used 50 μM curcumin since at this concentration curcumin completely suppressed the secretion of both IL-8 and IL-6. As observed in Fig. 3, addition of cadmium increased both IL-6 and IL-8 mRNA levels. Furthermore, both IL-6 and IL-8 mRNA responses to cadmium were blocked by curcumin (Fig. 3A and B). Finally, curcumin alone had no effect on basal levels of IL-6 and IL-8 mRNA transcript levels (Fig. 3A and B).

### 3.5. Curcumin prevents activation of Erk1/2 induced by cadmium

The results presented above clearly show that curcumin prevents secretion of IL-8 induced by cadmium in HBE cells (Fig. 4). Since inhibition of the MEK/Erk1/2 pathway blocked secretion of IL-8 induced by cadmium, we investigated whether curcumin affected the activation of Erk1/2 by cadmium. Addition of cadmium led to increased phosphorylation of Erk1/2 after 2 h (Fig. 4A). Pretreatment of HBE cells with curcumin for 1 h blocked the cadmium-induced activation of Erk1/2 (Fig. 4A).

### 3.6. Cadmium enhances NADPH oxidase activity in HBE cells

The NADPH- and NADH-dependent formation of $O_2^{\cdot-}$ by HBE cells under basal and cadmium-treated conditions was studied using the lucigenin chemiluminescence assay. At 50 μM concentration of NADPH the cadmium-treated cells showed a 4.7-fold increase in NADPH oxidase activity as compared to the untreated control cells (1950 ± 96 RLU vs. 9176 ± 668 RLU for untreated control cells and cadmium-treated cells, respectively). Conversely, NADH at the same concentration did not seem to affect lucigenin chemiluminescence in both control and cadmium treated cells (558 ± 46 RLU vs. 794 ± 41 RLU for untreated control cells and

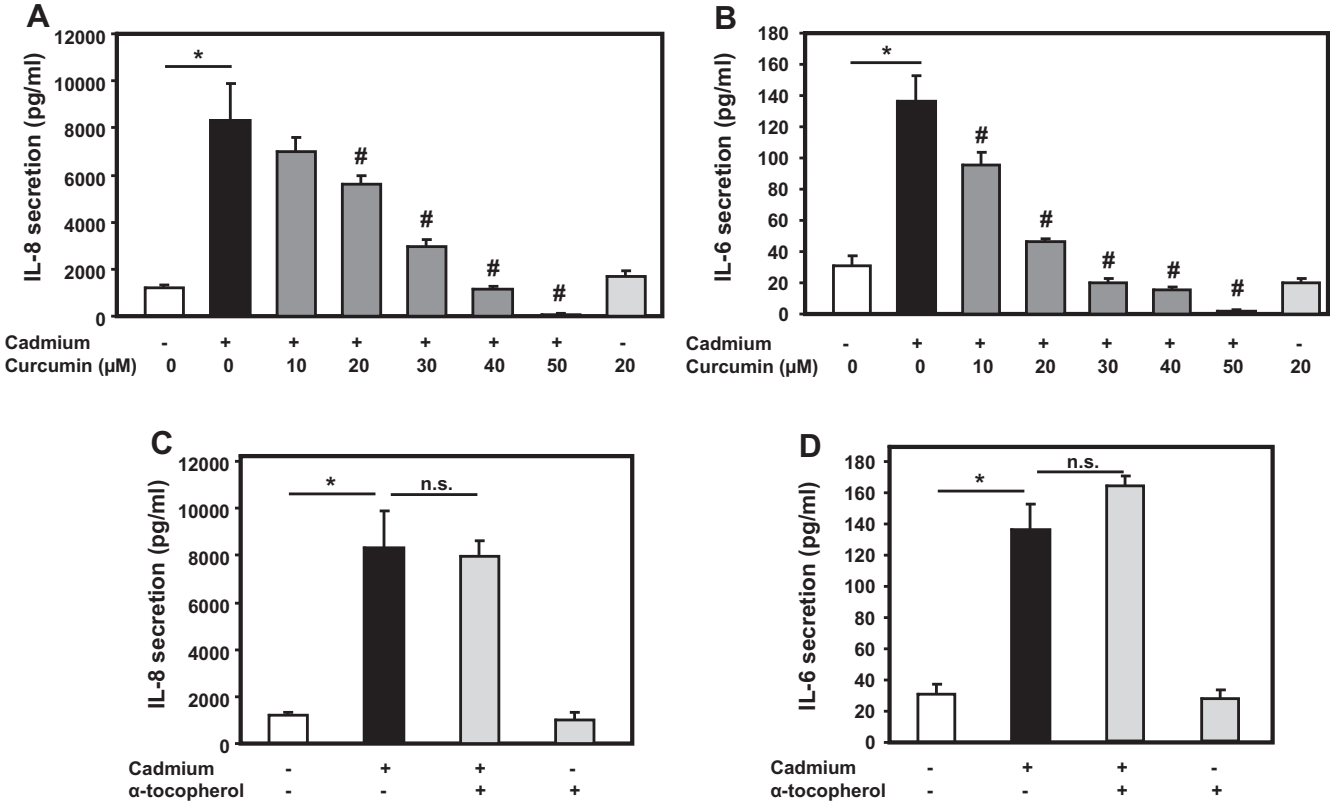


**Fig. 2.** Curcumin, but not α-tocopherol, prevents secretion of IL-8 and IL-6 induced by cadmium. HBE cells were treated with 50 μM cadmium and 10–50 μM curcumin (A and B), or 60 μM α-tocopherol (C and D). IL-8 (A) and IL-6 (B) present in the supernatants were measured by ELISA. Data are expressed as mean ± SE of at least three independent experiments. $^{*}p < 0.05$ when compared to control, $^{\#}p < 0.05$ when compared to cadmium treatment, n.s., not significant.

cadmium-treated cells, respectively). As expected, DPI (100 μM) inhibited the NADPH oxidase activity by nearly 2.5-fold in intact HBE cells. These results show that cadmium significantly enhanced NADPH oxidase activity.

### *3.7. Inhibition of NADPH oxidase prevents activation of Erk1/2 and secretion of IL-8*

Next, HBE cells were pretreated with the NADPH oxidase inhibitor DPI. Such treatment prevented phosphorylation of Erk1/2 in response to cadmium (Fig. 4B). Accordingly, HBE cells pre-treated with DPI showed significantly reduced secretion of IL-8 in response to cadmium (Fig. 4C). Conversely, DPI did not decrease secretion of IL-6 (Fig. 4D). Taken together, these results suggest that cadmium induces activation of the MEK/Erk1/2 MAPK pathway via activation of NAPH oxidase, and curcumin could prevent cadmium-induced activation of Erk1/2.

## 4. Discussion

Cadmium is a toxic metal that can be inhaled via particulate matter and cigarette smoke. Most of the studies on cadmium have focused on its toxicity and there have been even fewer studies on its inflammatory properties. Therefore we investigated the effect of subcytotoxic doses of cadmium on production of inflammatory cytokines by human airway epithelial cells. Here we show that the antioxidant curcumin, a natural inhibitor of NF-κB, can prevent the release of both IL-6 and IL-8 by airway epithelial cells in response to the toxic metal cadmium.

Cadmium inhalation has been linked to lung cancer and COPD, diseases characterized by chronic inflammation [4,5]. Recently,

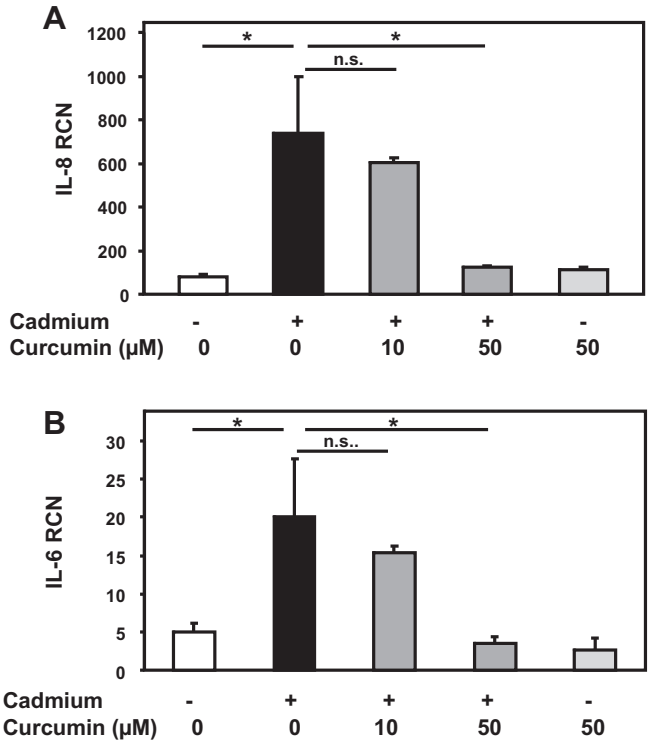


**Fig. 3.** Curcumin prevents up-regulation of IL-8 and IL-6 mRNA levels. HBE cells were treated for 4 h with cadmium in presence or absence of curcumin at the indicated concentrations. IL-8 and IL-6 mRNA transcript levels were measured by real time quantitative RT-PCR and expressed as relative copy numbers (RCN). Data are expressed as mean ± SE of at least three independent experiments. $^{*}p < 0.05$; n.s., not significant.

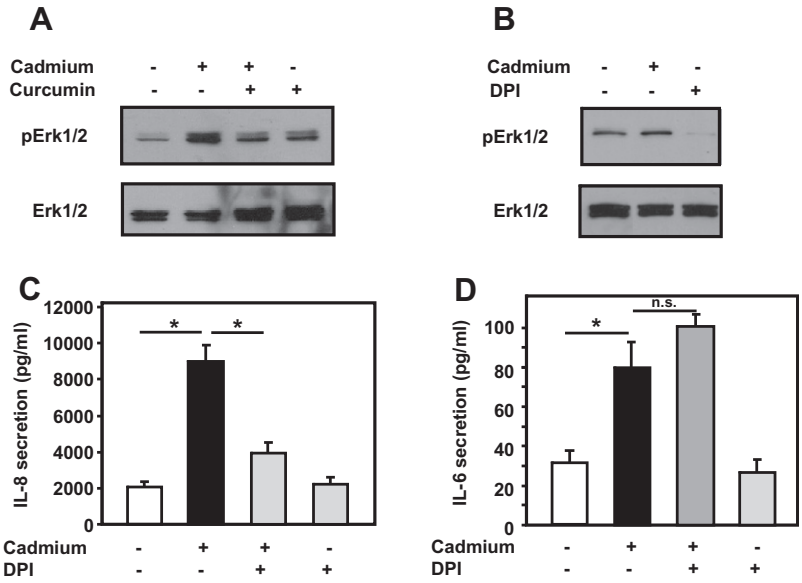


**Fig. 4.** Curcumin inhibits the cadmium-induced activation of Erk1/2 MAPK pathway. HBE cells were treated for 2 h with 50 μM cadmium and 50 μM curcumin (A) or 10 μM of the NADPH oxidase inhibitor DPI (B). The presence of activated Erk1/2 (pErk1/2) was detected by immunoblotting as described in "Materials and methods". The same amount of proteins was loaded in each lane. Data are representative of at least three independent experiments. (C and D) HBE cells were treated for 24 h with 50 μM cadmium and 10 μM of the NADPH oxidase inhibitor DPI. IL-8 (C) and IL-6 (D) present in the supernatants were measured by ELISA. Data are expressed as mean ± SE of at least three independent experiments. $^{*}p < 0.05$ and n.s., not significant.

cadmium was reported to promote IL-6 and IL-8 secretion by human intestinal epithelial cells, human type 2 epithelial cells, and alveolar macrophages [9,26]. Here we examined the effect of cadmium on cytokine secretion by airway epithelial cells. Since airway epithelial cells are polarized, it was important to establish whether the secretion of IL-6 and IL-8 in response to cadmium was polarized as well. Our data are in agreement with previous reports indicating that airway epithelial cells stimulated with IL-1β, TNF-α, or a cationic polypeptide, preferentially secrete cytokines into the apical compartment [27,28]. On the other hand, secretion of IL-8 towards the basal side has been demonstrated in other epithelial cells cell types [29,30]. It is therefore possible that the direction of the secretion depends on the cell-type and/or the nature of the stimulus.

Curcumin has previously been reported to have anti-inflammatory properties. Curcumin can block activation of NF-κB by inhibiting phosphorylation and degradation of inhibitor kappa B alpha [19,20]. NF-κB plays a critical role in the transcriptional regulation of various pro-inflammatory genes in various cells [31–33]. We made the interesting observation that cadmium-induced secretion of IL-8 is inhibited by the MEK/Erk1/2 inhibitor UO126 but not the NF-κB inhibitor Bay 11-7082. These results suggest that cadmium induces secretion of IL-8 via an Erk1/2-dependent pathway in airway epithelial cells. Interestingly, this work is the first report showing that curcumin can prevent both cadmium-induced IL-8 and IL-6 secretion, although different signaling pathways are involved in the production of these cytokines by airway epithelial cells. Curcumin can be degraded and therefore we cannot exclude that some of the effects observed are due to its degradation product(s) [34]. The AP-1 family of transcription factors can be activated by the upstream MEK/Erk1/2 pathway. Curcumin has been reported to inhibit AP-1 activation. Here we show that curcumin prevents phosphorylation of Erk1/2 in airway epithelial cells exposed to cadmium. Accordingly, it was recently reported that curcumin decreased basal phosphorylation of Erk1/2 in several lung carcinoma cell lines [35]. On the other hand, Chun et al. reported that curcumin inhibited the catalytic activity of Erk1/2 in mouse skin but had no effect on its phosphorylation upon treatment with the phorbol ester TPA [36]. Thus, it is possible that curcumin differently affects Erk1/2 activation in different cell types and depending on the anatomical location of the cells (lung vs. skin).

Cadmium is not a redox-active metal like iron, copper, or chromium but has been shown to stimulate the production of reactive oxygen species (ROS) [37,38]. It was recently shown that cadmium triggers production of ROS by inducing NADPH oxidase activity leading to induction of signaling pathways such as STAT3 [39]. Our data shows that addition of DPI reduces Erk1/2 phosphorylation and inhibits cadmium-induced secretion of IL-8 by HBE cells. These results suggest that cadmium activates MEK/Erk1/2 pathway via induction of NADPH oxidase and that activation of the MEK/Erk1/2 pathway by cadmium is downstream of induction of NADPH oxidase. However, DPI can also inhibit other electron transporters such as nitric oxide synthase and xanthine oxidase [40]. Taken together, these results suggest that cadmium induces secretion of IL-8 via production of ROS that is upstream of the MEK/Erk1/2 MAPK pathway, and curcumin could prevent cadmium-induced secretion of IL-8 by inhibiting activation of Erk1/2. The fact that the antioxidant curcumin prevented cadmium-induced secretion of both IL-6 and IL-8 by airway epithelial cells suggests that curcumin could be a good therapeutic agent to prevent airway inflammation due to cadmium inhalation.

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